REMARKS

Reconsideration and allowance are respectfully requested.

An Information Disclosure Statement cannot be filed at this time because the pending Action is final. But to update their disclosure requirement, Applicants submit the following four documents that were cited during prosecution of the related European Appln. No. 03810513.6:

1. Henricks et al. "Stress diminishes infiltration and oxygen metabolism of phagocytic cells in calves" Inflammation 11:427-437 (1987)
2. Kato et al. "Measurement of chemiluminescence in freshly drawn human blood. I. Role of granulocytes, platelets, and plasma factors in zymosan-induced chemiluminescence" Klin. Wochenschr. 59:203-211 (1981)
3. Mian et al. "Of stress, mice and men: A radical approach to old problems" in *Stress and Health: New Research* (K. Oxington ed.) New York: Nova Science Publ., pp. 61-79 (2005)
4. Thompson et al. "The effect of stress on the immune response of Atlantic salmon (*Salmo salar* L.) fed diets containing different amounts of vitamin C" Aquaculture 114:1-18 (1993)

If the Examiner would like to review the prosecution history of the related European application or the third-party observations filed therein, she is invited to contact the undersigned or to download the relevant papers from www.epoline.org.

Note that document 3 cited above is a review article by the inventors that was published after the effective filing date of this application and would assist in conveying the importance of their invention.

Claims 1-2, 5-14, 16-17 and 23-24 were rejected under Section 112, second paragraph, as being allegedly "indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." Applicants traverse.

In the Action, further clarification was alleged to be necessary to understand how (c) the comparison of "superoxide production above basal observed in said test whole blood sample with superoxide production above basal observed in a control whole blood sample as defined in (b) above under the same conditions" is performed. Both measure-

ments were made in accordance with the requirements of (b). Claim 1 sets out all the essential technical features of Applicants' invention. It is not seen that any change of wording is necessary or would clarify the claimed method.

The essence of the invention is application to whole blood samples of what might be termed a "challenge" employing an inducer (e.g., PMA) capable of stimulating superoxide production in neutrophils. There is need for a whole blood sample taken from an individual known, or suspected of, exposure to a psychological stressor (the test sample of claim 1) and a control whole blood sample as defined in claim 1(b). In each case (test and control), the inducer (e.g., PMA) is added and superoxide production can be monitored in real time relative to basal (same sample without inducer). It will be evident that a single control result might be used for comparison with many test samples. Claim 1(b) specifies determining superoxide production above basal in the test sample after addition of the inducer. The comparison is made with superoxide production in a control sample above basal after addition of the same inducer. This precisely equates with the requirement of claim 1(c).

As confirmed by the various examples, in test samples taken from individuals exposed to a stressful event, superoxide production above basal in the test sample is dampened compared with the control. This is accounted for by stress itself causing neutrophil activation and PMA-challenge test looking at residual capacity for superoxide production in such samples. It is this residual capacity for superoxide production which is equated with coping capacity for the stress factor of concern.

Real time monitoring of superoxide production above basal for test and control samples by chemiluminescence assay in response to PMA-challenge is illustrated by the examples. Claim 1 is consistent in indicating that comparison of superoxide production can be made at any time point at which the control sample exhibits elevated superoxide production in response to inducer challenge. Thus, referring to Figure 1 of Applicants' specification, 15 minutes after PMA-challenge test, the control samples from non-transported animals showed maximum superoxide production above basal by chemiluminescence assay. At the same time point, test samples from transported animals showed far less superoxide production above basal, this being indicative that neutrophil

capacity for superoxide production had been dampened by the stress arising from transportation.

Applicants request withdrawal of the Section 112, second paragraph, rejection because the pending claims are clear and definite.

Claims 1-5 (sic), 9-11 and 23-24 were rejected under Section 103(a) as allegedly unpatentable over Tsukamoto et al. (Jpn. J. Hyg. 49:827-836, 1994) in view of Pfefferkorn (U.S. Patent 5,492,816). Applicants traverse.

It is simply not possible to arrive at the claimed invention from Tsukamoto et al. This disclosure has far more in common with the studies reported in Ellard et al. (Intl. J. Psychophys. 41:93-100, 2001), which was cited in the background section of Applicants' specification on page 2, than the claimed invention. Thus, it is instructive to look initially at Ellard et al. (a copy is appended for ease of reference). Both Ellard et al. and Tsukamoto et al. rely on determining neutrophil activation by the NBT reduction assay technique. This is a histological staining technique which involves counting stained cells under a microscope (see page 95, column 1, lines 1-16, of Ellard et al.). This technique does not quantify the amount of superoxide produced. Using this technique, Ellard et al. showed that a short-term mental stressor causes an increase in the percentage of activated neutrophils. This study might be contrasted with Example 4 of Applicants' specification in which blood samples were taken from humans subjected to a mental stressor task, and then subjected to a PMA challenge test in accordance with the claimed invention to determine residual capacity for superoxide production. This experimental group showed significantly reduced responsiveness to PMA compared to controls; the degree of responsiveness for members of the experimental group being an indicator of coping capacity for the stressor. This is very different concept from the NBT assay of Ellard et al. Indeed an important feature of the invention is that whole blood samples can be used without reference to leukocyte/neutrophil numbers.

Like Ellard et al., Tsukamoto et al. use the NBT assay only to compare neutrophil activation in blood of mice in response to different caging conditions: Crowd–I control conditions (4 mice per cage) and Crowd–II conditions (16 mice per cage). It appears from the Action that the depressed superoxide production noted in section (3) of the

abstract for experiment one led to an assumption that Tsukamoto et al. had the same objective of Applicants' invention except that a different technique of determining superoxide production was used. This is wrong. Tsukamoto et al., like Ellard et al., scored neutrophil activation by the NBT assay technique. They were not looking at residual capacity for superoxide production. While the abstract refers to "depressed" superoxide production, Tsukamoto et al. were not in fact measuring superoxide production as such and moreover reference to the legend of Figure 4 of their paper reveals, "Although Crowd–II conditions tended to be depressed in each test, no significant difference was found" (emphasis added). This would seem to apply additionally when bacterial cells or endotoxin were added to the samples, presumably to simulate a bacterial infection (middle- and right-hand columns of Figure 4). Ellard et al. suggest that the Crowd–II conditions, if causing real stress compared to the lower crowding conditions, might have resulted in higher scoring of activated neutrophils. But the essential point to note is that Tsukamoto et al. were merely interested in cell numbers affected by suspected stressful caging conditions instead of measuring superoxide production.

Even if one considered carrying out the same experiment except with a chemiluminescence assay for superoxide production, this would not lead one of ordinary skill in the art to use a superoxide inducer in a challenge test. Indeed, this would destroy the objective of the experiment in Tsukamoto et al., which was to look at the effect of a suspected stressor alone (or with simulated bacterial infection) on neutrophil numbers and percentage activation.

Pefferkorn refers to PMA, but merely in the context of test cell systems for modifying of a conventional luminol chemiluminescence assay by addition of orthovandate salt. On page 4 of the Action, reference is made to PMA as "enhancing detection of superoxide anion" but PMA is not part of the detection system of a luminol assay. PMA is used to simulate neutrophils in one embodiment of the claimed invention. The basic concept of the invention is to determine whether the ability of neutrophils to respond to such challenge has been dampened by stress. Applicants discovered that whole blood samples may conveniently be employed without reference to neutrophil numbers (see the Amendment of December 8, 2006 from page 22, 2nd paragraph, to page 23, 3rd

paragraph). Thus, the invention is very different from what Tsukamoto et al. were doing in their experiment one and involves concepts which are in no way derivable from their studies even when the cited references are combined.

Claims 1-2, 12-14 and 16-17 were rejected under Section 103(a) as allegedly unpatentable over Tsukamoto et al. (Jpn. J. Hyg. 49:827-836, 1994) in view of Carlson et al. (U.S. Patent 6,319,953). Applicants traverse.

Applicants' claimed invention necessarily requires a challenge with a neutrophil-inducer in whole blood samples and measuring their superoxide production compared to basal without inducer. In this way, a measure of residual capacity for superoxide production in whole blood samples is obtained which enables very convenient, reliable quantification of stress without any need for time-consuming cell counting. As noted above, the failure of Tsukamoto et al. to teach or suggest these limitations are not remedied by Pfefferkorn. Here Carlson et al., who disclose a method of screening for stress-relieving drugs, also do not remedy the deficiencies of Tsukamoto et al. Therefore, the claims are not obvious over the combination of the cited references.

Withdrawal of the Section 103 rejections is requested because the invention as claimed would not have been obvious to one of ordinary skill in the art at the time it was made from the combination of Tsukamoto et al., and either Pfefferkorn or Carlson et al.

Having fully responded to all of the pending rejections contained in this Office Action, Applicants submit that the claims are in condition for allowance and earnestly solicit an early Notice to that effect. The Examiner is invited to contact the undersigned if any further information is required.

Respectfully submitted,

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STRESS DIMINISHES INFILTRATION AND OXYGEN METABOLISM OF PHAGOCYTIC CELLS IN CALVES

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Abstract—The influences of a stress situation on the phagocytic cell function before and after infection with *Pasteurella haemolytica* were measured in calves. No differences in phagocytic and metabolic activity of alveolar macrophages (AMs) were observed in vitro between cells isolated from stressed and nonstressed animals. The uptake of bacteria and the migration of polymorphonuclear leukocytes (PMNs) did not differ. However, the production of superoxide by PMNs isolated from stressed animals was significantly diminished as compared to control PMNs. The stressed and six of the nine control calves were then infected intrabronchially with *P. haemolytica*. Phagocytic cell function was again evaluated after three days. The lavage fluid obtained from the lungs of the infected animals contained about three times more PMNs and six times more AMs as compared to the lavage fluid of the control calves. However, the increase in phagocytic cell numbers was less by half in the calves infected after the stress period. No differences were detected in phagocytic and metabolic activity of PMNs and AMs among control, infected, and stressed and infected calves. However, the chemotactic activities of PMNs obtained from infected stressed and infected nonstressed animals were diminished as compared to control PMNs. In conclusion, the metabolic responsiveness of PMNs is diminished and the accumulation of phagocytic cells at a site of infection is reduced after a stress period.

INTRODUCTION

Bovine respiratory tract disease complex, known as shipping fever pneumonia, is one of the most important causes of disease and mortality in cattle. Initial viral infections and stress factors are thought to contribute to the facilitation of the clinical pattern. *Pasteurella* species, especially *P. haemolytica* type A1, are

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the principal causes of the advanced clinical signs, terminal lesions, and fatality (1). These bacteria produce toxins which are cytotoxic to phagocytic cells of bovines in vitro (2–5). However, previous exposures of the animals to a viral infection and/or a stress situation are necessary to induce experimentally an infection with *P. haemolytica* (6–9). Phagocytic cell function and mucociliary clearance can be depressed by viruses (9–12). Stressful conditions increase the plasma cortisol levels of bovines (8, 9) which impair phagocytic cell function and give rise to an increased susceptibility to bacterial infection (8, 13).

In the lung the primary defenders against inhaled particles and microorganisms are the alveolar macrophages (AMs). These cells exhibit a variety of biological activities, such as phagocytosis and killing of microorganisms, and secretion of enzymes, reactive oxygen metabolites, and arachidonic acid metabolites, such as prostaglandins and leukotrienes. During infections or inflammatory reactions other phagocytic cells, such as polymorphonuclear leukocytes (PMNs), can also be detected in the lung tissues which infiltrate from the blood. Although data exist on the effects of stress on the susceptibility of the host for infections with *P. haemolytica* (6–9) and on the effects of cytotoxins liberated by *P. haemolytica* on phagocytic cell function in vitro (2–5), there are almost no data concerning phagocytic cell activity during and/or after stress situations, followed by an infection. In our study we investigated the function of AMs and blood PMNs in calves, immediately after a stress period. The animals were also infected with *P. haemolytica* and the phagocytic cell defense was again evaluated three days after the start of the infection, with or without a previous stress period. It was found that stress influenced the oxygen metabolism of PMNs and the influx of phagocytic cells in the lung during infection.

MATERIALS AND METHODS

Animals and Experimental Design. Studies were performed on 15 clinically healthy dairy calves, approximately 5 months old and reared as described by Binkhorst et al. (manuscript in preparation). According to serum neutralization tests and lung lavage fluid cultures before and during the experiment, the calves were free of respiratory virus infections (bovine rhinotracheitis virus, parainfluenza-3 virus, bovine respiratory syncytial virus). All calves, except one control calf, showed serum neutralization titers against bovine virus diarrhea. The experiments were carried out in three successive series of five calves: one control calf (C), two calves inoculated with a *P. haemolytica* type A 1 strain (I), and another two calves inoculated with *P. haemolytica* immediately after a stress period (S+I). Stress was induced by forcing the calves to walk on a treadmill (speed 6 km/h) for 2 h and by keeping the calves soaking wet with cold water and leaving them in a cold airstream (15°C). After the stress period and lung lavage procedure, the calves in group I and S+I were inoculated with 15–20 ml *P. haemolytica* type A 1 [10^8–10^9 colony forming units (CFU)/ml] and again 3 h later with 10–15 ml bacteria suspension (10^9–10^{11} CFU/ml). Lung lavages for alveolar cell sampling and peripheral blood samples were taken immediately after the stress period. Lung lavage and blood collection were repeated three days after the start of the experiment.

Bacteria. P. haemolytica type A1 (generously supplied by Dr. N.J.L. Gilmour, Moredun Research Institute, Edinburgh, Scotland, U.K.) was grown in brain–heart infusion at 37°C. After 3 h, the suspension contained 10^8–10^9 CFU/ml and after 6 h, 10^9–10^{11} CFU/ml. These bacterial suspensions were used to infect the calves. *E. coli* strain PC 2166 was used for the quantitation of phagocytosis. This bacterium was grown overnight at 37°C in 5 ml Mueller-Hinton broth (Difco Laboratories, Detroit, Michigan) to which 0.02 mCi of [^{3}H]thymidine (specific activity 5 Ci/mmol; Amersham, England, U.K.) had been added. The desired concentrations were obtained spectrophotometrically after the bacteria had been washed three times and suspended in phosphate-buffered saline (PBS). Opsonization was achieved by incubating bacteria for 30 min at 37°C in a dilution of serum in Krebs–bicarbonate buffer. After incubation, the mixtures were centrifuged (15 min, 1600*g*), the supernatants were discarded, and the bacteria were resuspended in Krebs–bicarbonate buffer to 10^8 bacteria/ml. Nonopsonized bacteria were prepared in a similar fashion except for the omission of an opsonic source.

Serum. Serum obtained from the blood of 10–15 bovines was pooled and stored in 1-ml portions at −80°C (pooled bovine serum). Serum was thawed shortly before use and diluted to the final concentration of 0–1% (v/v) in Krebs–bicarbonate buffer.

Preparation of AM Suspensions. AMs were obtained by lavage of the lungs of the calves by fiberoptic bronchoscopy (Olympus CIF type XQ; PR 90 cannula). Each animal was placed in a box, and the head was immobilized. The fibroscope was introduced into a nostril and passed through the bronchi into the right diaphragmatic lobe, approximately 25 cm caudal to the bifurcation. Then 50–60 ml of a sterile phosphate-buffered saline solution (pH 7.4) was slowly injected under optical control and aspirated under vacuum. The procedure was repeated two to three times until ±100 ml lavage fluid was collected into plastic tubes. Cells were sedimented at 400*g* for 10 min at 4°C and resuspended in Krebs–bicarbonate buffer. The cell suspensions were carefully layered onto Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden). After centrifugation for 35 min at 240*g*, the monuclear cell layer at the interface was collected and washed twice with buffer. The final cell preparations contained 5×10^6 AMs/ml of Krebs–bicarbonate buffer. Viability was assessed by trypan blue exclusion. Characteristics of AMs and differentiation of cell types were measured by morphology.

Preparation of PMN Suspensions. Peripheral blood samples were collected by jugular vein puncture in heparinized tubes. Blood was centrifuged at 400*g* for 10 min, and the plasma was withdrawn. The cell pellet was resuspended in 40 ml 13.2 mM phosphate buffer (pH 7.2). After 50 sec, 20 ml of 13.2 mM phosphate–462 mM NaCl buffer (pH 7.2) was added and mixed. The blood cells were centrifuged (10 min, 240*g*) and resuspended in 24 ml Krebs–bicarbonate buffer. The cell suspensions were carefully layered onto 4 × 3-ml Ficoll-Paque. After centrifugation for 35 min at 240*g*, the mononuclear cell layer at the interface was removed. The PMN pellet was washed three times and the final PMN suspensions were adjusted to a concentration of 5×10^6 cells/ml of Krebs–bicarbonate buffer.

Quantitation of Phagocytosis. Phagocytosis was studied by using [^{3}H]thymidine-labeled bacteria (14). Samples of 0.2 ml of AM or PMN suspensions (5×10^6 cells/ml) were mixed with 0.2-ml aliquots of the suspensions of opsonized and nonopsonized *E. coli* (10^8 bacteria/ml). These mixtures were incubated in a shaking waterbath at 37°C for 2, 5, 15, or 30 min. After incubation, 2.5 ml of ice-cold PBS was added to stop the phagocytosis reaction. The AMs or PMNs, together with cell-associated bacteria, were separated from unassociated bacteria by centrifugation at 180*g* for 5 min at 4°C and by washing three times in cold PBS. AM or PMN pellets were solubillzed in scintillation liquid and radioactivity was measured in a liquid-scintillation counter. Total radioactivity was determined by centrifugation of AMs or PMNs with all bacteria at 1600*g* for 15 min. The amount of cell-associated radioactivity at each of the four time periods was expressed as the percentage of the total radioactivity that was available during the incubations.

Superoxide Anion Generation. Generation of superoxide anion was assayed by measuring the reduction of ferricytochrome *c* (horse heart type VI, Sigma Chemical Co., St. Louis, Missouri

by using a modification of the method described by Babior et al. (15). The reaction mixtures contained 2.5×10^6 AMs or PMNs, 90 μmol ferricytochrome *c*, and 2.5 mg zymosan (Sigma), preopsonized in 100% serum, or 1×10^6 AMs or PMNs, 90 μmol ferricytochrome *c*, and 10 ng phorbol myristate acetate (PMA; Sigma) in Krebs–bicarbonate buffer (final volume 1 ml). Paired reaction mixtures with and without superoxide dismutase (EC 1.15.1.1, bovine blood type I, Sigma; 100 μg/reaction mixture) were employed. All the compounds were added to biovials and shaken in a waterbath at 37°C. After incubation, the reaction mixtures were centrifuged at 4°C for 10 min at 740*g*. The difference in absorbance of the supernatant fractions was determined at 550 nm in a double-beam spectrophotometer. Nanomoles of ferricytochrome *c* reduced were determined from the increase in absorbance at 550 nm by using the extinction coefficient $E_{550nm} = 2.10 \times 10^4/M/cm$.

Chemotactic Activity. Chemotaxis of PMNs was determined using the under-agarose technique (16). PMN suspensions (5 μl; 5×10^7 cells/ml) were placed in one well (diameter 2.4 mm) that had been cut into agarose; another well (2.7 mm apart) contained 5 μl of pooled bovine serum. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air for 18 h. Migration distance was calculated as migration towards serum minus spontaneous migration in the opposite direction.

Statistical Analysis. Results have been expressed as the mean of three or more independent observation ± standard error of the mean (*SEM*). For significance analysis, Student's *t* test was performed. *P* values exceeding 0.05 were considered not significant.

RESULTS

Characteristics of Bronchoalveolar Lavage Fluids. During the lavage of the lungs of the calves, about 50–70% of the injected solutions were regained from the lung compartments. The cells in the bronchoalveolar fluids were mainly AMs and PMNs. Smaller amounts of lymphocytes (<5%) and epithelial cells (<2%) were also observed. The viability of the cells was 70–80%. The total numbers of AMs harvested from bronchoalveolar fluids of the control group and the stressed group were not different. A small increase in the amount of PMNs was detected in the fluids isolated from stressed animals compared to control animals (Table 1).

After the lavage procedure, the stressed animals and six of the nine control animals were infected intrabronchial with *P. haemolytica*. After three days, phagocytic cells were more numerous in infected calves than in control animals (Table 1). However, in the animals infected after a stress period, the increase of phagocytic cells was less by half compared to infected, nonstressed animals (Table 1).

Phagocytic and Metabolic Activity of AMs. The uptake of bacteria, opsonized in 0 or 1% serum, by AMs was determined in vitro. No differences in phagocytic capacity between the different groups of calves were observed, both on day 0 and day 3 (Figure 1).

To examine the metabolic activity of the AMs isolated from the bronchoalveolar lavage fluids of the different animals, these cells were incubated with

Table 1. Recovery of Phagocytic Cells from Bronchial Lavage Fluids of Calves[a]

Day	Group	Viability (%)	AMs ($\times 10^6$/ml)	PMNs ($\times 10^6$/ml)	N
0	C	72 + 3	15.8 ± 1.7	3.6 ± 1.0	9
0	S	74 ± 4	19.4 ± 5.1	8.8 ± 1.6[b]	6
3	C	81 ± 3	17.7 ± 3.4	9.6 ± 2.6	3
3	I	85 ± 2	58.0 ± 11.8[c]	60.8 ± 4.3[d]	6
3	S+I	81 + 2	30.0 ± 5.9	34.5 ± 3.7[d,e]	6

[a]The lavage procedure was performed on control (C) and stressed (S) animals on day 0 and on control (C), infected (I), and stressed and infected (S+I) calves on day 3. Data represent the number of cells determined in 100 ml lavage liquid.
[b]$P < 0.05$ compared to control values of the same day.
[c]$P < 0.02$ compared to control values of the same day.
[d]$P < 0.001$ compared to control values of the same day.
[e]$P < 0.001$ compared to infected values of day 3.

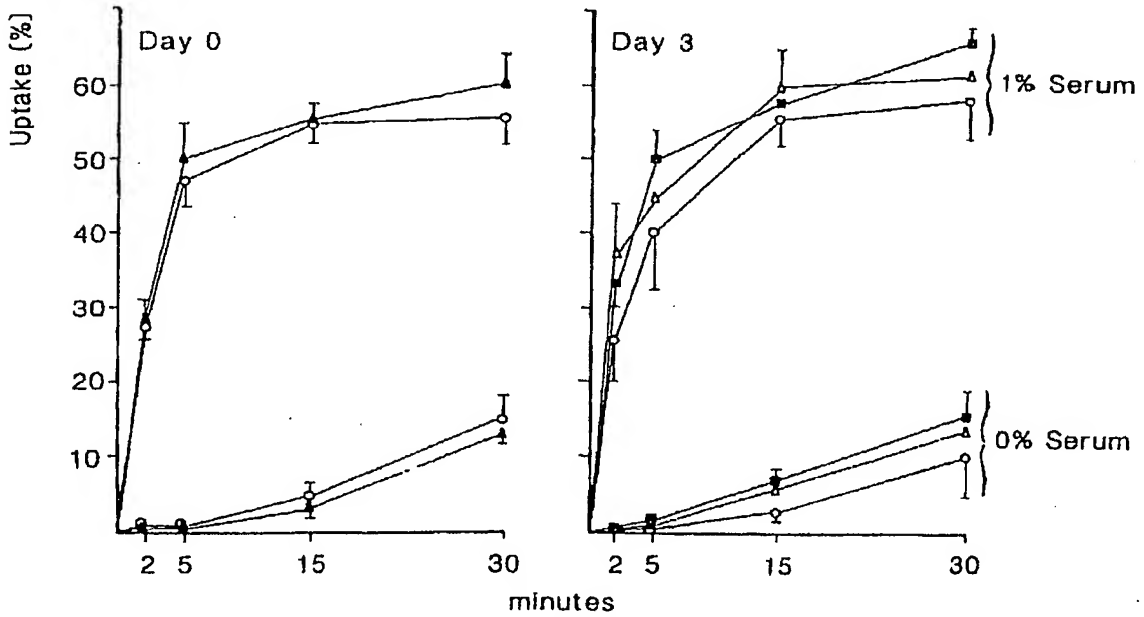

Fig. 1. Phagocytosis of *E. coli*, opsonized in 0 or 1% serum, by AMs isolated from control (○) and stressed (▲) animals on day 0 and from control (○), infected (■), and stressed and infected (△) animals on day 3. The 10^6 AMs were mixed with 2×10^7 radiolabeled bacteria and incubated for 2, 5, 15, and 30 min at 37°C. Unassociated *E. coli* were removed by washing and centrifugation. From the amount of cell-associated radioactivity, the uptake of bacteria was calculated.

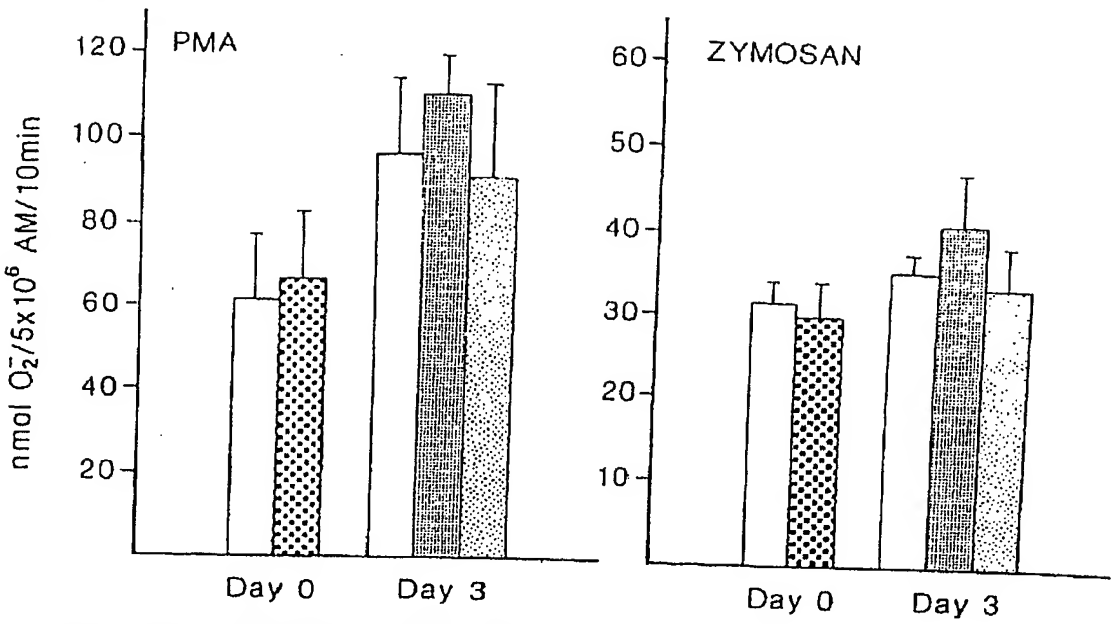

Fig. 2. Production of superoxide by AMs isolated from control (□) and stressed (▨) animals on day 0 and from control (□), infected (▩), and stressed and infected (▨) calves on day 3. The AMs were stimulated in vitro with PMA or opsonized zymosan. The 2.5 × 10^6 AMs were mixed with 2.5 mg zymosan or 1 × 10^6 AMs with 10 ng PMA. To the reaction mixtures 90 μM ferricytochrome *c* was added. The incubation took place for 10 min at 37°C. After removal of the AMs and the zymosan by centrifugation, the amount of superoxide produced was determined by measuring the absorbance of the reaction mixture at 550 nm.

different stimuli (opsonized zymosan and PMA) and the amounts of superoxide generated were detected by using ferricytochrome *c*. No differences were observed between cells of control and stressed animals on day 0 (Figure 2). Also on day 3 no significant differences in metabolic capacity existed between the cells obtained from the three groups of animals (Figure 2).

Functional Activities of Blood PMNs. From each animal, heparinized, venous blood was collected on day 0 and day 3 and PMNs were isolated and purified. The total numbers of PMNs isolated at day 0 were equal in the different groups of calves (2.4–3.7 × 10^7 cells/10 ml blood). The isolated PMNs were tested in vitro for several phagocytic cell activities. The uptake of opsonized and nonopsonized bacteria did not differ between PMNs isolated from stressed and control animals on day 0 (Figure 3). Also, no differences were observed in phagocytic capacity between the PMNs of the three groups of calves on day 3 (Figure 3).

The amount of superoxide generated by PMNs isolated from stressed animals was significantly diminished as compared to control PMNs (Figure 4). This decrease could be observed using both kinds of stimuli (PMA and zymosan) and amounted 20–45% ($P < 0.05$). On day 3, no differences were detected: PMNs isolated from infected and from stressed and infected animals

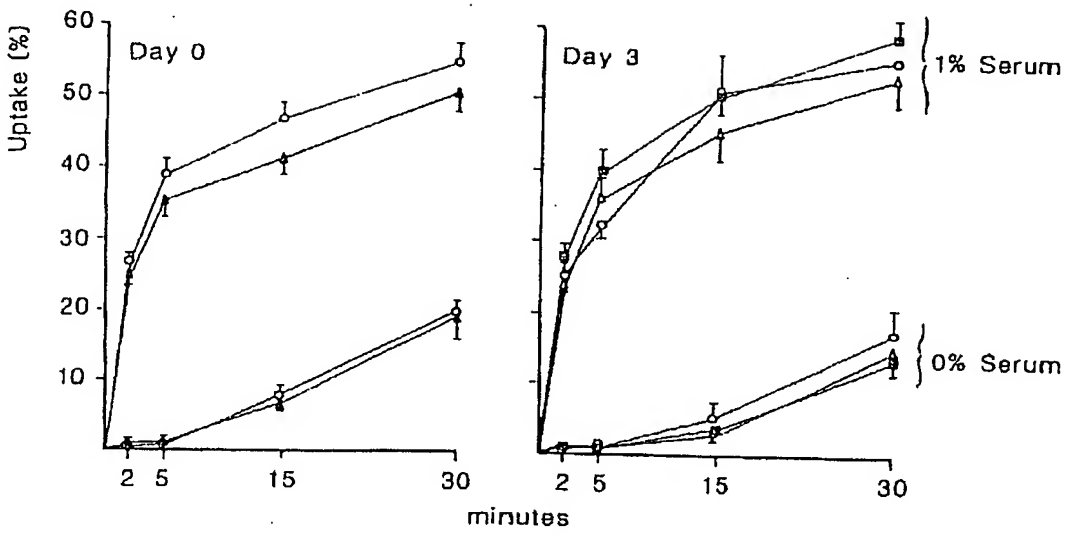

Figure 3. Phagocytosis of *E. coli* by PMNs isolated from control (○) or stressed (▲) calves on day 0 and from control (○), infected (▩), or stressed and infected (△) calves on day 3. PMNs (10^6 cells) were incubated with radiolabeled opsonized and nonopsonized *E. coli* (2×10^7 bacteria) for 2, 5, 15, and 30 min at 37°C. Unassociated bacteria were removed by washing and centrifugation, and the uptake of *E. coli* was calculated from the amount of PMN-associated radioactivity.

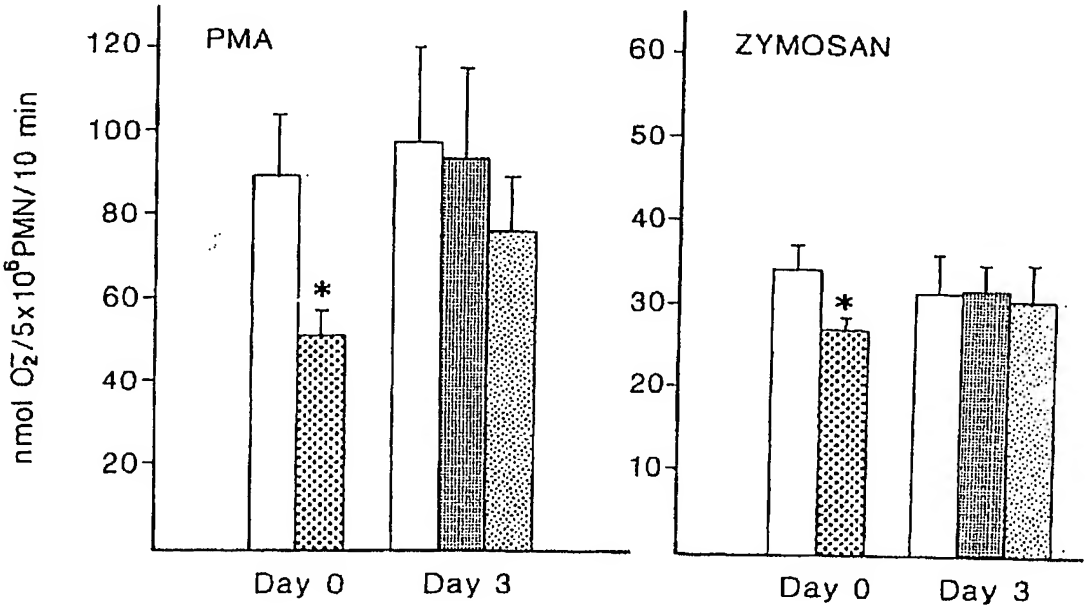

Figure. 4. Production of superoxide by PMNs isolated from control (□) or stressed (▨) calves on day 0 and from control (□), infected (▩), or stressed and infected (▨) animals on day 3 during stimulation with PMA or opsonized zymosan. The 2.5×10^6 PMNs were mixed with 2.5 mg zymosan or 1×10^6 PMNs were mixed with 10 ng PMA in the presence of 90 μM ferricytochrome *c* and incubated for 10 min at 37°C. The PMNs and the zymosan were removed, and the absorbance of the supernatant fraction was measured at 550 nm to determine the amount of superoxide produced. $^*P < 0.05$ compared to control values.

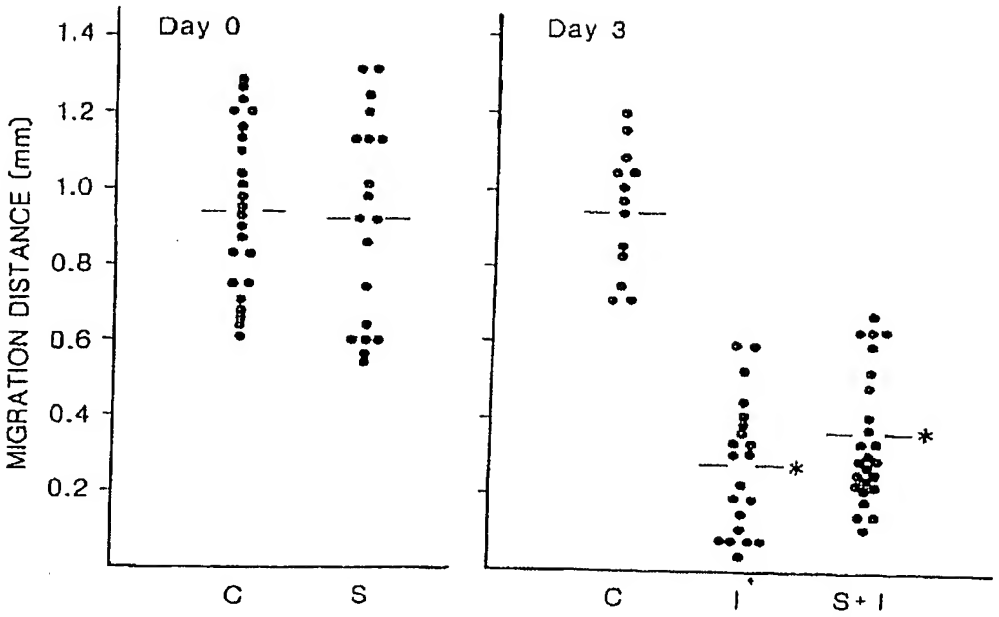

Fig. 5. Chemotaxis of PMNs isolated from control (C) or stressed (S) calves on day 0 and from control (C), infected (I), and stressed and infected (S+I) animals on day 3. Chemotaxis was performed under agarose and using activated serum as attractant at 37°C for 18 h in a humidified atmosphere of 5% CO_2–95% O_2. Migration distance was measured microscopically in millimeters, and directed migration was calculated as migration towards activated serum minus spontaneous migration. *$P < 0.001$ compared to control values.

produced as much superoxide as PMNs obtained from control animals (Figure 4).

The chemotactic response of the PMNs was assayed under agarose towards activated serum. PMNs obtained from stressed animals migrated equally well as control PMNs at day 0 (Figure 5). On day 3, a decrease in chemotactic activity was observed in PMNs obtained from infected (stressed and non-stressed) animals compared to control PMNs (Figure 5).

DISCUSSION

Bovine respiratory disease is considered to be caused by the interaction of the animals with environmental factors, such as viral infections and/or stress: transportation, handling, cold, crowding (1, 6–9). These factors may influence or alter normal host defense against bacteria, resulting in an enhanced susceptibility of the animals for infections with *Pasteurella* species, especially *P. haemolytica*. However, the exact mechanisms of how stress factors could influence host defense mechanisms is unknown. Investigation to the effects of stress factors, such as cold or transportation, are mainly performed on very young calves (8, 17). In these animals, a short-lasting increase in plasma cortisol levels is

found (8, 17). In older calves (6–8 months old), handling results in elevated plasma cortisol levels which remain above normal for at least three days post-challenge (9). In our study, older calves were used (5–6 months old), so plasma cortisol levels could be expected to be increased for several days.

Cortisol is a naturally occurring glucocorticoid which has antiinflammatory and immunosuppressive actions. When synthetic glucocorticoids, like dexamethasone, are administred in vivo to cattle, PMN oxidative metabolism, cytotoxicity, and ingestion are supressed in vitro (13). The set-up of Roth and Kaeberle's investigation (13) is similar to the set-up of our experiments: increasing natural corticosteriod levels by dexamethasone administration instead of stress and testing phagocytic cell function in vitro to evaluate the effects of the handling on the phagocytic cells. The metabolic activity of the PMNs was decreased in stressed animals compared to nonstressed animals, which could be caused by enhanced levels of glucocorticoids. No effects on chemotactic and phagocytic cell activity were observed. In contrast, Roth and Kaeberle found decreased ingestion of bacteria and enhanced random migration of PMNs after dexamethasone administration (13). Possible explanations for these discrepancies may be the difference in age of the animals and/or the differences in potencies of action of dexamethasone and cortisol.

Besides cortisol levels, plasma levels of catecholamines are also increased in response to stress (18). In general, these hormones influence cell function by interaction with adrenergic receptors, present on the cell membranes, which results in altered cyclic-AMP levels intracellularly. Some data on cyclic-AMP metabolism and phagocytic cell function are present in the literature. Phagocytic cell responses are depressed by exogeneous cyclic-AMP and agents that elevate intracellular cyclic-AMP levels (19). Also, β-adrenergic receptors have been characterized on rat peritoneal macrophages (20) and β-adrenoceptor agonists can modulate the release response of these cells (21). The decreased metabolic activity of PMNs after a stress period could be caused by increased cyclic-AMP levels in these cells due to stimulation of β-adrenoceptors by enhanced levels of adrenaline or noradrenaline in the blood stream of the calves. No effects of stress on the uptake of particles was observed. In previous studies (22), we also did not observe effects of cyclic-AMP-inducing agents on phagocytosis of PMNs. The stress situation did not alter the metabolic and phagocytic responsiveness of the AMs of the calves. This observation supports the hypothesis that circulating factors in the blood, which do not reach the AMs present in the bronchoalveolar space, are involved in the changes in PMN function. In a following experiment, we will try to quantitate the levels of cortisol and catecholamines in the circulation of the different groups of calves.

In the second part of our study, the animals were infected intrabronchially with *P. haemolytica*. After three days, increased numbers of AMs and PMNs were harvested from the lungs of the infected animals. However, the accumu-

lation of phagocytic cells in stressed animals was less by half compared to nonstressed animals. The accumulation of PMNs and macrophages at a site of infection is necessary for host resistance. Bassett and Tait (23) demonstrated a reduction in the total numbers of leukocytes migrated into the peritoneal cavity of stressed rats injected with glycogen. This phenomenon is probably due to an increased adhesiveness of PMNs in stressed animals (24). Increased PMN adherence is associated with a decreased ability of PMNs to migrate (25), resulting in fewer phagocytic cells accumulated at the site of an infection.

In conclusion, we found metabolic responsiveness of PMNs diminished by stress and reduced numbers of PMNs and AMs accumulated in the lungs of stressed, infected animals. These phenomena could contribute to the development of *P. haemolytica* infections during stress situations in the animals.

Acknowledgments—The authors thank Ms. Helen van der Vliet and Ms. Betty van Esch for their excellent technical assistance.

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Measurement of Chemiluminescence in Freshly Drawn Human Blood

I. Role of Granulocytes, Platelets, and Plasma Factors in Zymosan-Induced Chemiluminescence

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Summary. The present investigations were undertaken to find out whether chemiluminescence measurements of stimulated granulocytes can be carried out in freshly drawn blood and – because of the ease of the method – be introduced into routine diagnostics.

Blood was drawn from the cubital vein of healthy volunteers at various times and under various conditions. Subsequently the zymosan induced and luminol amplified chemiluminescence was recorded and analyzed. It could be demonstrated that variations existed between individuals which can, however, be minimized when photon counts obtained under standard conditions were related to the number of granulocytes present in the blood samples. It could be further demonstrated that also platelets are activated by zymosan as well and that they, contribute to the total chemiluminescence by a share of about 5%. Platelet chemiluminescence can effectively be suppressed by aspirin. Opsonising factors in plasma (presumably antibodies and/or complement) play a decisive role in the intensity and kinetics of blood chemiluminescence. Measurements of zymosan induced chemiluminescence in freshly drawn unfractionated and fractionated blood seem to be especially suited to monitor and analyze deviations and defects of the cellular and humoral defence mechanisms.

Key words: Chemiluminescence in whole blood – Granulocyte chemiluminescence – Platelet chemiluminescence – Opsonins

Chemilumineszenz-Messung in humanem Vollblut

I. Die Rolle von Granulozyten, Blutplättchen und Plasmafaktoren bei der Zymosan-induzierten Chemilumineszenz

Zusammenfassung. Die vorliegenden Untersuchungen bilden die Grundlage, um die im Prinzip sehr einfache Messung der Chemilumineszenz stimulierter Granulocyten in frisch gewonnenem Vollblut durchzuführen und damit für die einfache Routine-Diagnostik anwendbar zu machen.

Gesunden Freiwilligen wurde zu verschiedenen Zeiten und unter verschiedenen Bedingungen kubital Venenblut entnommen und anschließend die durch Zymosan induzierte und durch Luminol verstärkte Chemilumineszenz analysiert. Die Chemilumineszenz-Kurven verschiedener Individuen zeigten Variationen, die jedoch verringert werden, wenn die unter Standardbedingungen gemessenen Photonen auf die Zahl der im Blut vorhandenen Granulocyten bezogen werden. Es zeigte sich ferner, daß Blutplättchen ebenfalls durch Zymosan aktiviert werden und zu etwa 5% an der gesamten Chemilumineszenz beteiligt sind. Die Plättchen-Chemilumineszenz läßt sich indes durch Zusatz von Aspirin nahezu vollständig ausschalten. Von entscheidender Bedeutung für die Intensität und Kinetik der im Vollblut gemessenen Chemilumineszenz ist die Anwesenheit opsonierender Plasma-Faktoren (Antikörper und/oder Komplement).

Die Messung der Chemilumineszenz im Vollblut erscheint uns geeignet, sowohl Abweichungen der zellulären wie der humoralen Abwehrfunktionen rasch und einfach zu erkennen.

Schlüsselwörter: Chemilumineszenz in Vollblut – Granulocytenchemilumineszenz – Blutplättchenchemilumineszenz – Opsonine

Since the discovery that intact granulocytes emit photons during the phagocytosis-induced metabolic respiratory burst [1], a multitude of work has been performed in order to clarify the nature and interrelationship of these two phenomena. By now, it is well established that granulocytes do respond to exterior stimuli with an increase in univalent reduction of molecular oxygen which leads to the generation of superoxide

anion (O_2^-). Superoxide anion can act either as a reductant or an oxidant and superoxide derived reactive oxygen species appear to be responsible for the granulocytes (oxygen dependent) bactericidal activity and also for the generation of chemiluminescence (CL) [2, 3, 5, 7, 8, 15, 20, 23, 30]. Thus, measurements of CL have been widely used for the detection and characterization of metabolic and functional granulocyte defects [4, 16, 29, 32].

In most studies reported so far, purified and washed granulocytes have been used, and special care was taken to avoid contamination with erythrocytes or hemoglobin which quenches or absorbs most of the generated photons. This implied that prior to testing the cells underwent time-consuming procedures and finally expressed functions which possibly did not mirror the original in vivo situation. Mention, however, has been made by various authors that granulocyte function in vivo, i.e. in blood or in the microenvironment of inflammatory sites is greatly modified by the presence of other cells, by mediators generated from platelets during blood clotting or by antibodies, immune complexes and complement components [9, 11, 12, 14, 17, 19, 33, 35, 39]. In order to study cellular functions under natural conditons, an attempt was made to analyze CL in freshly drawn unfractionated blood [38]. We here report our first results.

Materials and Methods

Chemicals and Media

Zymosan (lot nor. DZ-3185) was obtained from Becton Dickinson (Orangeburg, N.Y.), Liquemin from Roche Pharmaceuticals Inc., sodium heparinate from Serva (Heidelberg, FRG) and sodium citrate-5,5-hydrate from Merck (Darmstadt, FRG).

Luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) was purchased from Sigma (München, FRG) and lipopolysaccaride (LPS, Novo-Pyrexal) was a gift of Dr. C. Galanos (Freiburg, FRG). Lysylacetylsalicylate (Aspegic) was obtained from Laboratoire Egic (Amilly, France).

Dulbecco's modified Eagle's medium containing HEPES (50 mM, pH 7.4) (and lacking $NaHCO_3$) was used for dilution of blood samples and for resuspension of cells (Eagle HEPES medium, EHM).

Luminol was rendered soluble in phosphate buffered saline (PBS) in the following way: luminol (2 mg) was suspended in PBS (1 ml) and triethylamine (8 µl) was added. After shaking and ultrasonication the solution was passed through a Millipore filter (0.45 µm) and stored in the dark at −20 °C.

Zymosan was suspended in EHM at a concentration of 50 mg/ml.

Blood Samples

Venous blood was drawn from the cubital vein of normal human donors who had not taken aspirin or indomethacin for a minimum of 21 days previously. Heparin (Liquemin) was generally used as an anticoagulant (10 IU/ml). Before use, heparinized blood samples were kept at 0–4 °C. For CL measurements, heparinized blood (0.1 ml) was diluted with Eagle HEPES medium (0.4 ml).

Granulocytes in blood and platelets in platelet-rich plasma were counted in a Neubauer chamber using novocaine (3%) for platelet and acetic acid (3%) for granulocyte counting.

Absorption of Plasma with Zymosan

Fresh heparinized blood was centrifuged (250 g, 5 min) and the supernatant (platelet-rich plasma, PRP) centrifuged again at higher speed (1,400 g, 10 min). To the supernatant, representing essentially cell free plasma (0.1 ml), graded amounts of zymosan (50 to 1,000 µg, suspended in 0.5 ml EHM) were added (total volume 0.6 ml). This mixture was incubated 40 min, 37 °C) and after centrifugation (1,400 g, 10 min), subjected to CL analysis.

Chemiluminescence Assay

For CL measurements, a Biolumat (model LB 9500, Berthold, Wildbad, FRG) was used in a semidarkened room. The sample to be analyzed was mixed with luminol (10 µl), pipetted into a round-bottomed vial (1 ml), placed into the Biolumat counting chamber (37 °C), and background CL was recorded. After 10 min, zymosan (in general 500 µg, in 10 µl EHM) was added. CL was continuously monitored using a Servogor recorder (model 210, BBC Goerz). Counts per minute (cpm) were plotted simultaneously by an interface connected Hewlett Packard calculator (model 97S).

Results

CL of Fresh Blood

Fresh venous blood (1 ml) was drawn from human volunteers, mixed with heparin (Liquemin, 10 IU/ml) and diluted with Eagle's HEPES medium (EHM, 4 ml). To an aliquot (0.5 ml), luminol (10 µl) was added and the mixture (in a 1 ml vial) placed into a Biolumat counting chamber (37 °C). After 10 min, zymosan (500 µg, 10 µl) was added and CL monitored continuously.

CL was first evident approximately 5 min after the addition of zymosan; it reached a maximum after 18–28 min and thereafter declined (Fig. 1 A). Occasionally, a small peak occurred 5–8 min after zymosan addition. If luminol was omitted from the blood sample, CL was not seen (Fig. 1 B).

Blood samples obtained from different donors ($n = 15$) showed variations in their light emission particularly at peak CL (18–28 min after zymosan addition (Fig. 2). On the other hand, no difference or only slight differences in zymosan-induced CL were seen with samples of one donor obtained on different days (data not shown). Also, only minor differences were seen in CL between blood samples taken in the morning ($6.4 \times 10^5 \pm 0.48 \times 10^5$ cpm) and in the afternoon ($5.8 \times 10^5 \pm 0.75 \times 10^5$ cpm; 5 donors and 15 measurements in both cases).

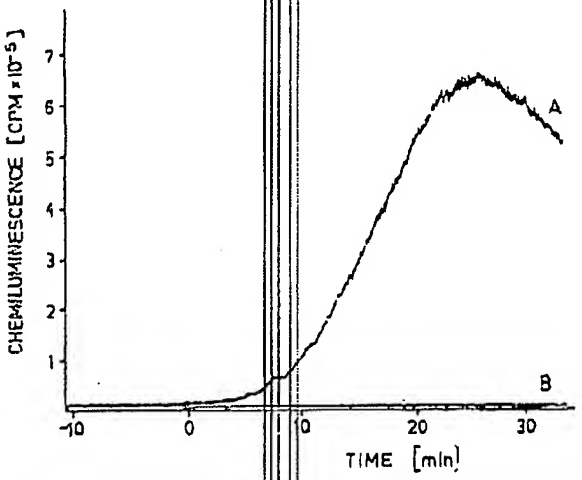

Fig. 1. Actual record of zymosan-induced chemiluminescence of freshly drawn human venous blood (*A* with luminol, *B* without luminol)

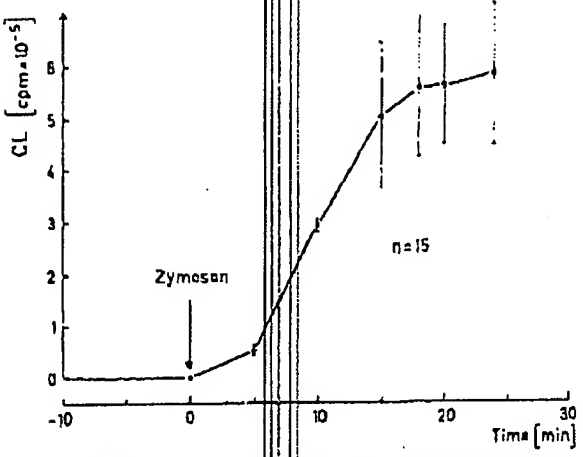

Fig. 2. Variation of zymosan-induced chemiluminescence of fresh blood obtained from 15 human volunteers

Blood samples of individual donors, however, varied greatly in their granulocyte counts. Since granulocytes have previously been shown to emit CL on incubation with zymosan [1], it was tentatively assumed that granulocytes were largely responsible for fresh blood CL. Therefore, the results described in Fig. 2 were expressed as cpm $\times 10^{-3}$ per 10^3 granulocytes (Fig. 3). The dramatic decrease of standard deviations calculated from individual samples suggests that granulocytes may indeed play a prominent role in zymosan-induced CL of fresh blood.

This assumption was supported by experiments involving blood samples from donors in which granulocytosis had been induced by intracutaneous injection of bacterial lipopolysaccharide (LPS). Blood samples were taken from donors before and 4 h after

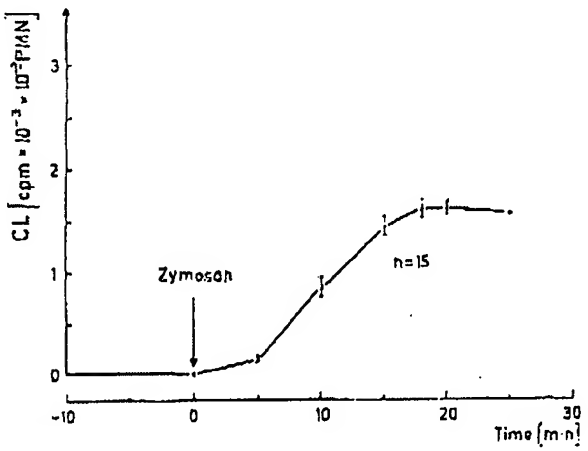

Fig. 3. Zymosan-induced chemiluminescence of fresh human blood; relation to granulocyte counts; PMN = polymorph nuclear neutrophils

Table 1. Zymosan-induced chemiluminescence and granulocyte counts in fresh blood from control and lipopolysaccharide (LPS) pretreated human donors

Experiment no.	Granulocytes		Chemiluminescence		Chemiluminescence	
	before LPS	after LPS	before LPS	after LPS	before LPS	after LPS
	10^{-6} cells/ml		cpm × 10^{-5}		cpm × 10^{-3} × 10^{-3} Granulocytes	
1	2.0	5.6	4.34	11.13	2.17	1.99
2	5.3	7.8	9.22	13.30	1.74	1.70
3	2.4	5.0	4.11	8.59	1.71	1.72

LPS treatment (6 × 0.2 µg) and both granulocyte counts as well as the zymosan-induced CL were estimated (Table 1). In three experiments, LPS induced a significant granulocytosis, and blood samples from leukocytotic donors exhibited a greatly enhanced CL response (Table 1). However, if this response (cpm) was expressed relative to 10^3 granulocytes a comparable value in samples of both normal and LPS-treated donors was obtained (without LPS: 1.87 ± 0.26 cpm × 10^{-3} PMN; with LPS: 1.80 ± 0.16 cpm × 10^{-3} PMN; mean of 3 experiments). This result again suggests that in zymosan-induced CL of fresh blood granulocytes play an essential role (see below).

Dependency of CL on Heparin Dose

In the experiments discussed so far, Liquemin had been added to fresh blood as an anticoagulant in

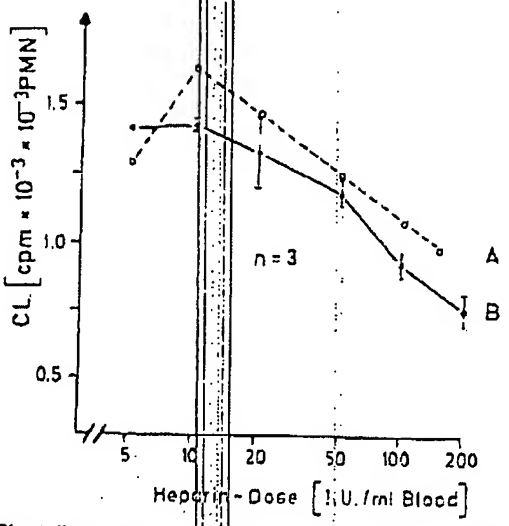

Fig. 4. Dependency of zymosan-induced peak chemiluminescence in fresh human blood on the heparin dose

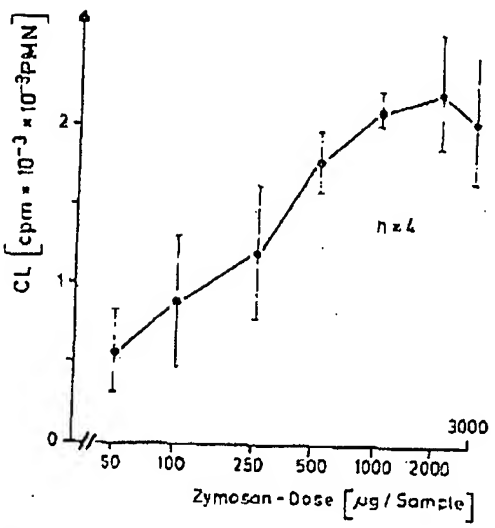

Fig. 5. Dependency of zymosan-induced peak chemiluminescence in fresh human blood on the zymosan dose

a final concentration of 10 IU/ml. To test its possible modifying influence on zymosan-induced CL, graded doses of Liquemin (5–200 IU/ml) were added to blood samples and, after zymosan (500 µg) addition CL was measured. In Fig. 4 the dependency of peak CL (expressed as cpm $\times 10^{-3} \times 10^{-3}$ PMN) on the Liquemin dose is shown. It is evident that with 10 IU/ml, CL is maximally expressed while smaller (5 IU/ml) and particularly larger doses suppress CL (Fig. 4B). A similar dose dependent inhibition of CL was seen using sodium heparinate (Fig. 4A). This latter finding shows that suppression of CL by Liquemin was not due to possibly present radical scavengers such as phenol, but was an intrinsic property of heparin. The experiments further demonstrate that the use of a heparin dose of 10 IU/ml is most suitable since this amount prevents coagulation, yet does allow maximal expression of CL.

The influence of sodium citrate (3.8%) on CL was also tested. If citrate blood (pH 7.4) was diluted and tested in the same way as heparinized blood, a CL response was obtained which was comparable in kinetics and intensity to that observed (see Fig. 1) with heparinized blood (data not shown).

Dependency of CL on Zymosan Dose

Diluted fresh blood samples (4 donors) were treated with graded amounts of zymosan (50–3,000 µg) and the peak CL estimated. It was found that already 50 µg of zymosan induced a significant CL response and that an almost linear dose response relationship existed up to an amount of 1,000 µg of zymosan (Fig. 5). With the largest dose tested (3,000 µg), CL was not maximally expressed. It should be noted that with increasing amounts of zymosan the maximum of CL appeared at earlier times relative to the time of zymosan additon ($\Delta t \approx 25$ min, 50 µg zymosan; $\Delta t \approx 17$ min, 1,000 µg of zymosan). Since 500 µg of zymosan induced a CL response within the linear portion of the dose response relationship (upper region, Fig. 5) this dose was used in most of the experiments described in this communication.

Fractionation of Fresh Blood

In order to elucidate the blood components involved in the mediation of zymosan induced CL, blood (containing Liquemin) was fractionated as shown in Fig. 6. Fresh heparinized blood was centrifuged (250 g, 5 min) and the supernatant (platelet rich plasma, PRP) as well as the sediment (S) containing leukocytes and erythrocytes were collected. PRP was further centrifuged at higher speed (1,400 g, 10 min). The sediment consisted mainly of (99%) thrombocytes (Th) and the supernatant represented essentially cell free plasma (P). The different fractions obtained were then tested individually and in various combinations for their capacity to respond with CL on incubation with zymosan.

CL of Blood Components

The leukocyte-erythrocyte sediment (S, 50 µl) from 1 ml blood (containing approximately 5.4×10^6 gran-

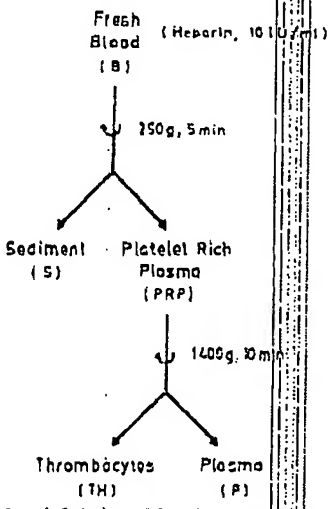

Fig. 6. Scheme of fractionation of fresh heparinized human blood (*B*) into sediment (*S*, leukocytes, erythrocytes), platelet rich plasma (*PRP*), thrombocytes (*TH*) and plasma (*P*)

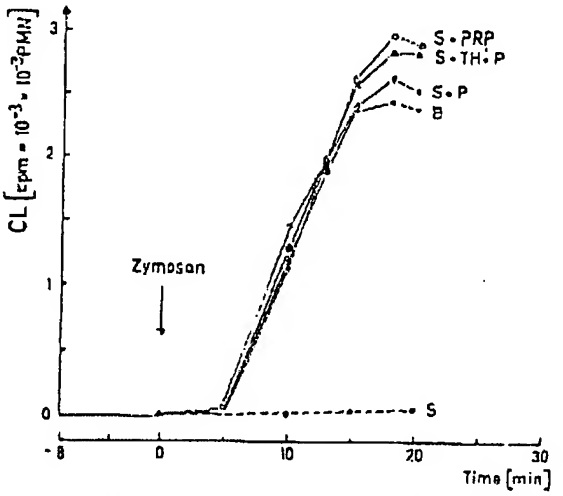

Fig. 7. Zymosan-induced chemiluminescence of fresh human blood (*B*) and combined blood components. For identity of blood components compare Fig. 6

ulocytes per ml) was suspended in EHM 0.45 ml) and after the addition of zymosan (500 µg) and luminol (20 µg) CL was measured. As compared to unfractionated blood samples (Figs. 1 and 2) a different CL response was obtained, peak CL occurred only after 60 min, and the intensity of CL was significantly lower (2.6×10^5 cpm = 933 cpm × 10^{-2} PMN). Also plasma generated a very poor CL response to zymosan. This residual reactivity was completely lost after passing fraction P through a Millipore filter (0.45 µm) (Fig. 8). If, however, sediment (50 µl) and plasma (50 µl + 0.4 ml EHM) were combined, a zymosan-induced CL response was observed which was comparable in kinetics and peak intensity to that seen with unfractionated blood (Fig. 7). This finding shows that cells present in the sediment (i.e. granulocytes) are largely responsible for the emittance of light induced by zymosan in blood. It further shows that for this activity a humoral factor is required which is present in plasma. As expected, addition of PRP (50 µl + 0.4 ml EHM) or thrombocytes (50 µl, 5×10^6 cells) plus plasma (50 µl + 0.35 ml EHM) to the sediment (50 µl) also gave rise to full zymosan-induced CL (Fig. 7).

Isolated thrombocytes (10^7 cells per 0.1 ml + 0.4 ml EHM) or plasma alone, on incubation with zymosan showed little or no CL, respectively, (Fig. 8). A mixture of thrombocytes (10^7 cells) and plasma (50 µl) however, responded to zymosan (500 µg) with CL which exhibited a maximum after 8–10 min (Fig. 8). A similar response was seen when PRP (0.1 ml + 0.4 ml EHM) was incubated with zymosan (Fig. 8). In both cases, however, the peak intensity of the CL response was low ($4–5 \times 10^5$ cpm/sample);

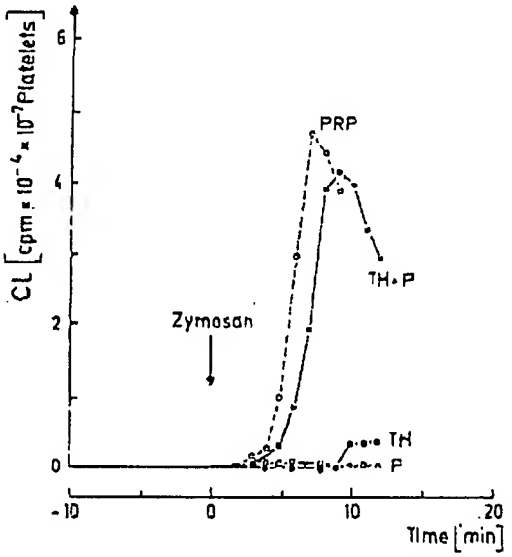

Fig. 8. Zymosan-induced chemiluminescence of isolated human thrombocytes (*TH*), plasma (*P*), platelet rich plasma (*PRP*) and a combination of thrombocytes plus plasma (*TH + P*)

it amounted to approximately 7% of that seen with unfractionated blood (6.5×10^5 cpm/sample; Figs. 1 and 2) and varied widely among different individuals and between samples from one donor obtained on distinct days.

These findings show that (to a minor extent) thrombocytes contribute to zymosan-induced CL of whole blood, and that for the expression of platelet activity a humoral (plasma) factor is also required. In addition, the fact that PRP (and thrombocyte plus plasma)-dependent CL had a maximum at 8–10 min

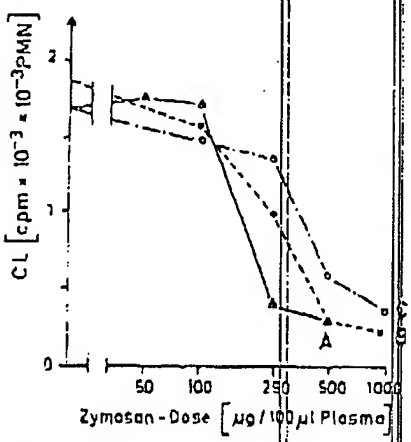

Fig. 9. Zymosan-induced chemiluminescence of fresh human blood-derived leukocyte sediment treated with zymosan-absorbed plasma. Shown is the peak CL response with 3 distinct plasma samples (*A, B, C*) absorbed with graded amounts of zymosan

suggests that the early peak occasionally seen in the CL response of whole blood (Fig. 1) is due to thrombocytes.

Absorption of Plasma with Zymosan

The experiments discussed above demonstrate that both granulocytes and thrombocytes emit CL on incubation with zymosan. This activity is fully expressed only if plasma is present. In order to study further the requirements of soluble factors, the following experiments were performed.

Samples of plasma (0.1 ml) obtained from fresh blood of 3 individuals were incubated with graded amounts of zymosan (50–1,000 µg in 0.5 ml EHM, 40 min, 37 °C). After centrifugation (1,400 g, 10 min), the supernatant (0.3 ml absorbed plasma (AP) was added to freshly prepared sediment (50 µl + 150 µl EHM) and the CL response to zymosan monitored. A greatly diminished CL was found in mixtures in which the plasma component had been absorbed with zymosan doses greater than 250 µg (Fig. 9). If absorption was carried out with 1,000 µg of zymosan, the peak CL observed was only approximately 15% of that seen with non-treated plasma in the 3 samples tested. This result shows that the plasma factors involved in the generation of leukocyte CL bind to zymosan and thus can be removed from plasma. Furthermore, the fact that different doses of zymosan are required to decrease peak CL response to 50% (Fig. 9) indicates that in the individuals analyzed the factors are present in different concentrations. It is worthwhile to note that the factors could also be removed by zymosan from fresh blood as well as from serum (data not shown).

Table 2. Influence of acetylsalicylate and indomethacin on zymosan-induced chemiluminescence of human fresh blood and platelet rich plasma

Additions	Chemiluminescence			
	Fresh blood	No. of samples	Platelet rich plasma	No. of samples
	% CL from Control ± SEM		% CL from Control ± SEM	
None	100	8	100	8
Acetylsalicylate[a]	108 ± 9.4 (n.s.)[b]	8	65.9 ± 5.7[c]	8
Indomethacin	110 ± 9 (n.s.)	5	94 ± 8 (n.s.)	5

[a] Lysyl derivative (Aspégic)
[b] n.s. = not significantly different from control
[c] $p < 0.0005$ (relative to control)

Effect of Aspirin and Indomethacin on Blood CL

CL (zymosan-induced) has been linked to cyclooxygenase activity [25] and therefore the influence of acetylsalicylic acid (lysyl derivative, Aspégic) and indomethacin on zymosan-induced CL of fresh blood was investigated. To fresh blood samples (0.1 ml + 0.4 ml EHM) lysylacetylsalicylate (20 µg/ml) or indomethacin (20 µg/ml) respectively, were given, incubated for 10 min, and after the additon of zymosan CL was monitored. It was found that the CL response of samples treated with cyclooxygenase inhibitors was not significantly different from that of controls indicating that cyclooxygenase activity does not greatly contribute to fresh blood CL (Table 2). CL of PRP, however, could be partly (34%) suppressed by acetylsalicylate; indomethacin in the doses used had no suppressive effect (Table 2). These results suggest that in the induction of CL of granulocytes, cyclooxygenase products do not participate while in thrombocyte-dependent CL such products appear to play a role.

Effect of Sodium Cyanide on Blood CL

In order to characterize the mechanisms involved in blood CL, the effect of sodium cyanide on zymosan-induced CL of fresh blood was tested. Blood samples were treated with graded amounts (0.1–10 mMol) of NaCN; zymosan was added 10 min later and the CL response determined. It was found (Fig. 10) that larger NaCN doses (5 and 10 mMol) decreased CL relative to controls by 75 and 90%, respectively (Fig. 10). Doses of 0.1 and 1 mMol, however, inhibited CL only slightly (7 and 14%, respectively). Since

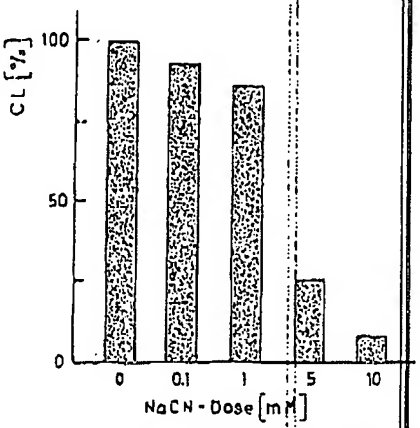

Fig. 10. Influence of sodium cyanide (*NaCN*) on zymosan-induced chemiluminescence of fresh human blood

these latter doses are known to block cellular oxidative phosphorylation it appears that the generation of fresh blood CL does not depend on respiratory chain activity.

Discussion

We report in the present study that measurements of zymosan-induced chemiluminescence can be performed in freshly drawn non-fractionated blood when luminol is present. In the absence of luminol, practically no photons can be measured because most of the light, emitted by granulocytes while in contact with and phagocytizing zymosan, is in the range between 540–640 nm [5] and is quenched or absorbed by erythrocytes. The addition of luminol has two highly beneficial effects: The number of photons is enhanced by two to three orders of magnitude and most of the light emitted will peak at 450 nm, a spectral range where hemoglobin interference is less marked.

Our results show that the CL curves obtained from the same individuals at different times are fairly reproducible when measurements are begun either immediately after blood sampling, or after a time interval during which blood has been kept cooled (0–4 °C). Differences in CL intensity, which were obvious when different individuals were compared, became much less pronounced when the actual cpm were related to the number of granulocytes present (expressed as cpm $\times 10^{-3}$ granulocytes). The obvious correlation between the number of granulocytes and photon counts was further strengthened by experiments in which leukocytosis had been induced by bacterial LPS. Again, it was shown, that the great bulk of CL evoked in blood by addition of zymosan is due to the stimulation of granulocytes.

In an attempt to clarify the role of platelets and of factors present in plasma, separated blood fractions and recombinations there of were studied. This analysis seemed of importance since platelets, like granulocytes show an immediate and striking increase in oxygen consumption [18] when stimulated by thrombin, collagen and also by zymosan [28]. As first described by Mills et al. [25] under these conditions platelets emit CL when luminol is present. Although platelet CL is weak as compared to granulocyte CL (based on cell numbers), it might nevertheless become significant when measurements are performed in blood. Our analyses have shown that the CL curves obtained after addition of zymosan to fresh blood of several individuals exhibited a small peak after 5–8 min. This peak proved to be platelet borne. Reports have shown that platelets can be stimulated by zymosan [24, 6]. The recombination studies described in the present paper revealed that in freshly drawn blood, or in samples kept on ice and then rewarmed immediately before testing, platelet activation and CL plays only a minor role; less than 10% of the total photon counts during the first 30 min are due to platelets. It should be mentioned, however, that this share of CL increases when blood is analyzed after periods of standing at room temperature or at 37 °C. Under these conditions, i.e. in the presence of platelets and plasma, granulocyte CL also increases with time [22]. This phenomenon points to a platelet-granulocyte interaction in standing blood [26, 13]. At present, we are engaged in a detailed CL analysis of the various types of granulocyte-platelet interactions which have been described in the early and recent literature [10, 21, 26, 27, 37].

In context with platelet-granulocyte interaction one additional observation seems to be of importance: platelet CL appears to be partly dependent on cyclooxygenase activity. This is indicated by our finding that aspirin, which is known to irreversible inactivate cycloocygenase [31] caused a significant depression of CL in PRP. A few contaminating granulocytes may possibly contribute to the remaining activity.

We therefore suggest that in clinical trials with a particular aim of studying granulocyte CL in blood the assays should be carried out in the presence of Aspirin. In this way, undesired side effects which occur when blood samples are standing at room temperature can be avoided.

Finally, our experiments have shown that plasma factors play a major role in both granulocyte and thrombocyte dependent CL. These activating factors can be removed from plasma by absorption with zymosan; their nature, however, remains to be elucidated.

In summary, our results show that zymosan-induced CL can be monitored in freshly drawn blood and that it is dependent on both cellular and humoral components. By recording the essential metabolic patterns which reflect the functional capacity of the body's most potent defensive cells in a medium which is close to the natural environment, CL is able to monitor the highly complex interplay between phagocytes, thrombocytes and plasma factors. Zymosan-induced CL of unfractionated blood is rapid and simple and may become a valuable method for clinical routine diagnostics.

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Chapter 3

OF STRESS, MICE AND MEN: A RADICAL APPROACH TO OLD PROBLEMS

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ABSTRACT

The tremendous destructive capabilities of reactive oxygen species in stress related disorders has become apparent only recently, although in early historical times the ancients may have been aware of the devastating power of stress on well-being. This chapter explores ancient myths and modern techniques surrounding stress-induced immunosupression in species as diverse as mice and humans, investigating techniques and mechanisms, and speculating on possible therapeutic interventions.

CURSES AND PLAGUES: FACT OR FICTION?

A modern idiom speaks of people making themselves sick with worry. As with so many aphorisms that are casually used, this linkage of stress and illness embodies a deep truth, a truth that may already have been familiar to the ancients.

It is an archaeological fact that most tombs of the Pharaohs had a curse written above the door, to deter intruders, warning that whoever opened or entered the tomb would die.

'Death will slay with his wings whoever disturbs the peace of the Pharaoh.'

(Budge 2001)

For centuries such curses have been dismissed as superstitious nonsense, but recent evidence shows that unwittingly or otherwise, the ancient Egyptians had harnessed a powerful tool: danger whether real or perceived can have devastating effect psychology can have on the immune system. Psychological stress may reduce the effectiveness of the immune system, thus increasing the risk of infection or disease (Dhabhar *et al.*, 1996; Kang *et al.*, 1996). The ancient Egyptians may have in fact been familiar with this. In 1999 the German microbiologist Gotthard Kramer analysed 40 mummies and found them contaminated with several varieties of potentially dangerous mould spores, which when exposed to air and a suppressed immune system could have caused fatal illness (Viegas 1999).

It is also tempting to speculate that the plague of boils mentioned in Book of Exodus 9:8-12, in the Old Testament, the sixth plague to hit Egypt would have been exacerbated by the psychological endured by those who had already experienced plagues of blood, gnats, flies and diseases of livestock. The psychological stress would have burdened only the Pharaoh and his people, thus automatically excluding those who firmly they were divinely protected.

Stress and Disease

Epidemiological studies show that those individuals who are more psychologically stressed are more prone to opportunistic infections (Clover 1989; Galinowski, 1997). For example stress associated with family dysfunction is significantly associated with increased incidence of upper respiratory tract infection and influenza B (Clover 1989) and work- related stress results in DNA damage in female workers (Irie 2001). Similarly persistent stress in elite athletes has been associated with chronic immunosupression and hence susceptibility to opportunistic infections (for review see Gleeson 2000). In animal models of stress, the spread of *Candida albicans* (an opportunistic fungal disease) is greater in stressed rats than non-stressed animals (Rodriguez *et al.*, 2001). Results from studies associating psychological stress with an increased cancer are contradictory: some suggest an increased risk of developing cancer in those exposed to psychological stress (Irie et al 2001) others support no such link (Johansen et al 1998)and most are inconclusive (Kiecolt-Glaser 1999). Overall psychological and behavioural factors may well influence the progression of cancer through psychosocial influences on immune function.

Current Measures of Stress

Objective, quantitative and practicable measures of stress are pivotal to studies in many branches of vertebrate biology, including animal welfare (e.g. Dawkins, 1980; Bateson & Bradshaw, 1997). The stress response in animals is currently assessed using a variety of techniques, including cortisol measurement (e.g. Palme & Möstl, 1997; Creel, 2001), haematological values (e.g. Millspaugh *et al.*, 2000) and behavioural observations (reviewed by Rushen, 2000). We have recently reported a novel technique to measure the stress response (McLaren *et al.*, 2003), based on the capacity of circulating neutrophils to produce reactive oxygen species in response to an external stimulus..

Neutrophils as Indicators of Stress

Neutrophils can respond rapidly to a wide range of physical and psychological stressors, and these responses can affect the ability of the immune system to react to ongoing or potential challenge (Maes *et al* 1999; Dhabhar *et al.*, 1995; Gleeson & Bishop, 2000). Stress has been shown to influence the number, distribution and activation of neutrophils in the blood in a rapid and reversible manner (Dhabhar *et al.* 1995; Goebel & Mills, 2000; Ellard *et al.*, 2001; McLaren *et al.*, 2003; Montes *et al.*, 2003; Mian *et al.*, 2003). One component of the response of neutrophils to stress is the release of reactive oxygen species (Mian *et al.*, 2003; Montes *et al.*, in press). This response is strictly controlled; only a subpopulation of neutrophils is activated, and the size of the subpopulation is related to the intensity of the stressor (Montes *et al.*, in press).

Leukocyte Coping Capacity: Stress Revealed from a Drop of Blood

Leukocytes are exposed to diverse factors: endocrine factors in the plasma, changes in blood biochemistry, changes in red cell haemodynamics, cytokines and factors released from other cells both circulating and non-circulating cells such as endothelial cells and changes in the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system. As stress affects each of these factors, leukocytes make ideal indicators of stress, being constantly exposed to a diverse range of stress stimuli.

We have used this fact to develop a novel approach to measuring stress. In summary, after a potentially stressful event, the capacity of circulating leukocytes to produce reactive oxygen species *in vitro* in response to challenge by phorbol myristate acetate (PMA) is measured, a measure called Leukocyte Coping Capacity (LCC). LCC is affected directly and rapidly by stress and the strengths of this technique, is: (1) the method can be used on whole blood (avoiding centrifugation); (2) it yields results within minutes and does not require baseline data from animals which have not been stressed and (3) it is practical to use in a wide variety of situations. Whole blood is used instead of fractionated centrifuged leukocyte subsets, as the primary aim is to produce a measure of cell activation that minimises *in vitro* manipulation of fresh blood, and to reflect as far as possible the *in vivo* condition of the cells. This method is of particular use in situations where a rapid assessment of the individual's ability to cope is required. The term LCC is used since a wide range of immune cells can respond to PMA, although neutrophils are responsible for most of the PMA response (Mian *et al.*, 2003), and represent the majority of leukocytes in the circulation. The aim of this chapter is to elucidate the mechanisms involved in the science underpinning this novel measure of stress.

The Leukocyte Coping Capacity reveals significant information about an animal's physiological status during and after stressful events (McLaren *et al.*, 2003). We have used it to demonstrate that even short-term psychological stressors can produce demonstrable physiological changes in neutrophil activation (Figure 2.1; Ellard *et al.*, 2001; McLaren *et al.*, 2003; Montes *et al.*, 2003; Mian *et al.*, 2003; Montes *et al.*, in press). For example, as a model

for studying stress we used wild badgers (*Meles meles*) that were trapped and transported (10 minutes on a trailer pulled by an all-terrain quad bike) as part of a long-term ecological and behavioural study (Macdonald & Newman, 2002). Badgers that had been transported showed, in comparison to badgers that had not been transported, changes in circulating cell numbers and composition that were indicative of stress (McLaren *et al.*, 2003), and their circulating neutrophils showed a reduced responsiveness to PMA (McLaren *et al.*, 2003; Figure 2.1).

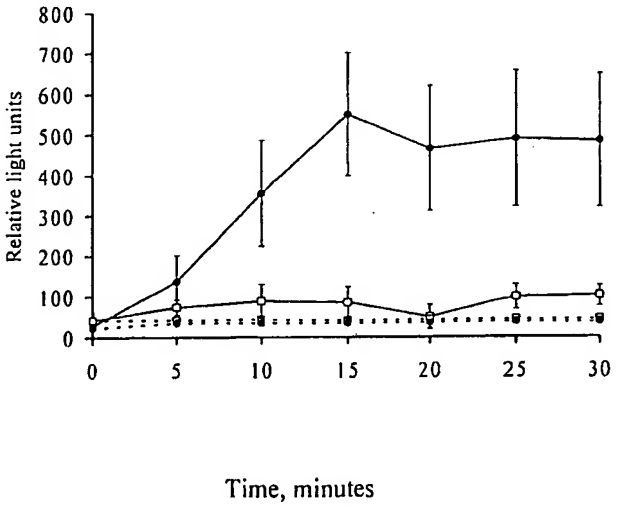


Figure 2.1 The response of circulating neutrophils to PMA (10^{-6} mol l^{-1}) stimulation in transported (lower solid line, n=8) and non-transported (upper solid line, n=8) badgers. Dashed lines represent unstimulated leukocyte activity levels (without PMA). The release of reactive oxygen species (measured here in relative light units) in response to PMA stimulation is much reduced by transport stress . That is LCC is diminished (McLaren *et al.* 2003).

The Importance of Reactive Oxygen Species

Reactive oxygen species (ROS) have an important role in immune defence, but can also potentially damage healthy tissue and organs (Weiss, 1989; Boxer & Smolen, 1998) and the activation of neutrophils is potentially detrimental to health (Weiss, 1989). Activated neutrophils and other phagocytic leukocytes can take up oxygen and make a range of ROS, such as $O^{\cdot}_2$, H_2O_2 and $OH^{\cdot}$, and these oxygen metabolites are potent microbicidal agents (Figure 1; Bokoch 2002). The potential for ROS to cause cellular damage is perhaps best demonstrated by the extensive antioxidant defence systems that are possessed by cells (Halliwell 1996; Kruidenhier & Verspaget 2002). Atanackovic et al., also reported (2003) reported that acute psychological stress reduced the capacity of human phagocytic leukocytes to produce basal levels of ROS. PMA used by McLaren et al (2003) measures the *capacity* of circulating neutrophils to release reactive oxygen species. Stress-induced immune alterations occur more or less in parallel to increases in hormonal & immunological markers of cells

(Atanackovic et al 2003) but immune alterations remained long after these traditional measures of stress (changes in heart rate and blood pressure) had returned to basal values (Mian *et al.* 2003).

WATCHING A HORROR FILM: STRESS BY PROXY

Even short-term psychological stressors can produce demonstrable physiological changes in heart rate, blood pressure and the activation of neutrophils (Ellard *et al.*, 2001; Goebel 2000). Activated neutrophils release many mediators, which can potentially damage even healthy tissue and organs, so this activation of neutrophils is potentially detrimental to health (Weiss, 1989; Weiss *et al.*, 1989; Boxer *et al.*, 1998). The many biologically active compounds released by neutrophils when they are activated include: cationic proteins, myeloperoxidase, lysozyme, acid hydrolases, lactoferrin (an iron-binding protein), B12-binding protein, cytochrome b and collagenase (Abramson, 1993).

Individuals who are more psychologically stressed are more prone to opportunistic infections (Clover 1989; Galinowski, 1997).

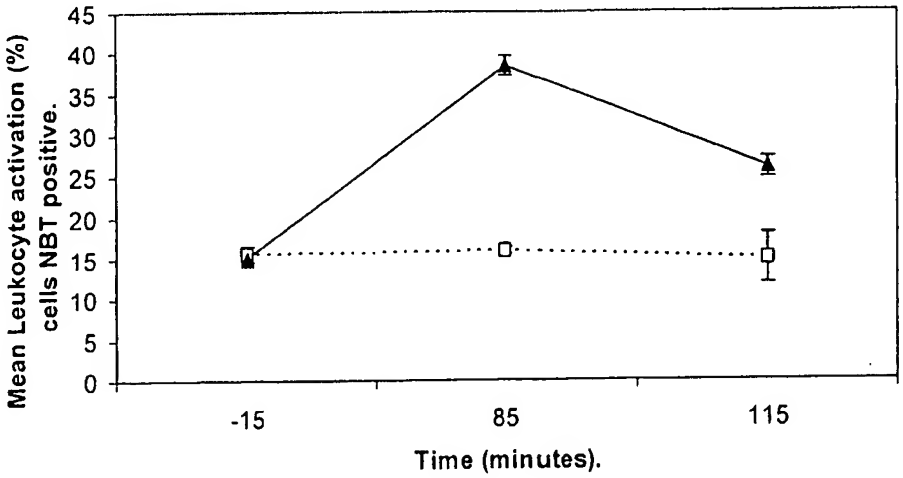
Acute psychological stressors have also been shown to increase the number of circulating leukocytes, and significantly to affect erythron variables such as haematocrit, mean cell haemoglobin content and the number of red blood cells (Maes *et al* 1998).

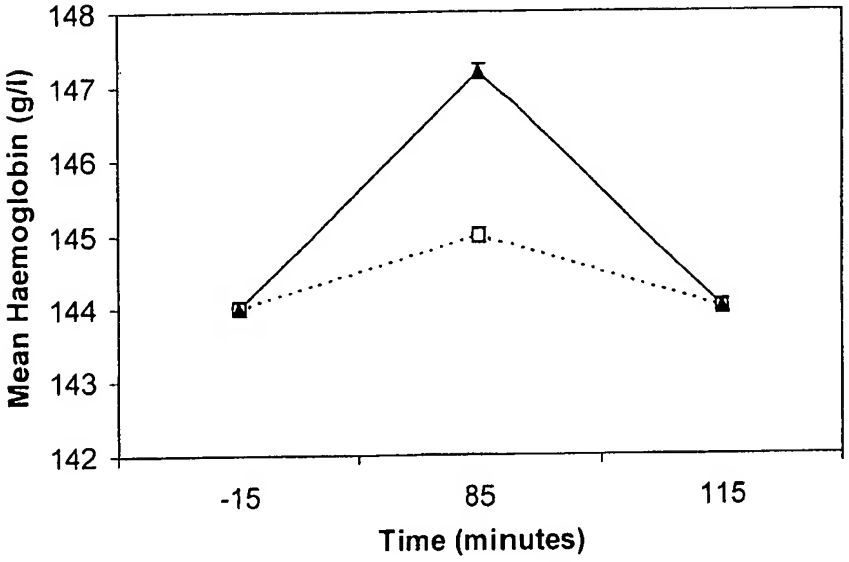
Psychological stress comes in many guises. Some individuals deliberately expose themselves to a form of psychological stress by watching horror movies.

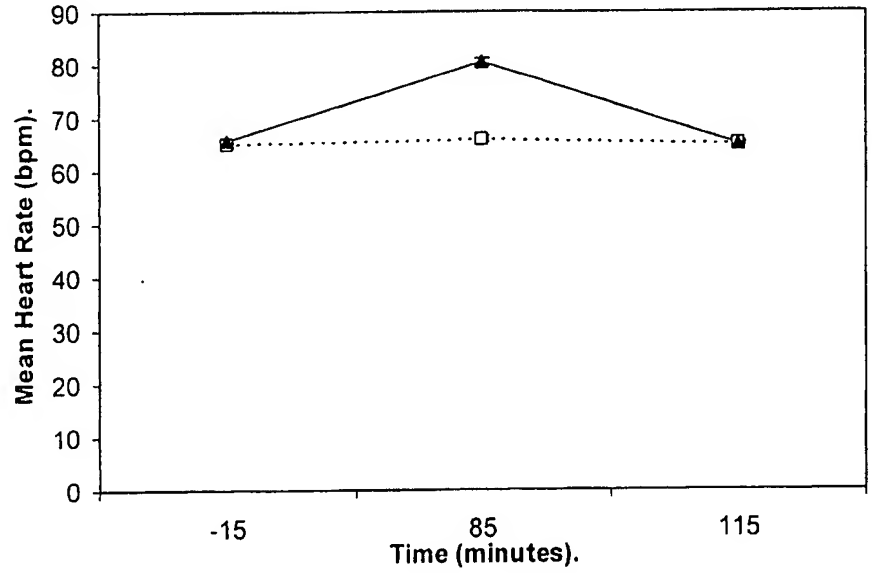
There have been no reported studies on the effect such a 'passive stressor' has on leukocyte activation. We asked whether passive observation of a stressful event, namely witnessing an emotionally disturbing movie, would result in leukocyte mobilisation and activation. The aim of this study was thus to investigate the effect of *observing* a stressful event, albeit fictitious, on leukocyte distribution and activation in otherwise healthy human subjects.

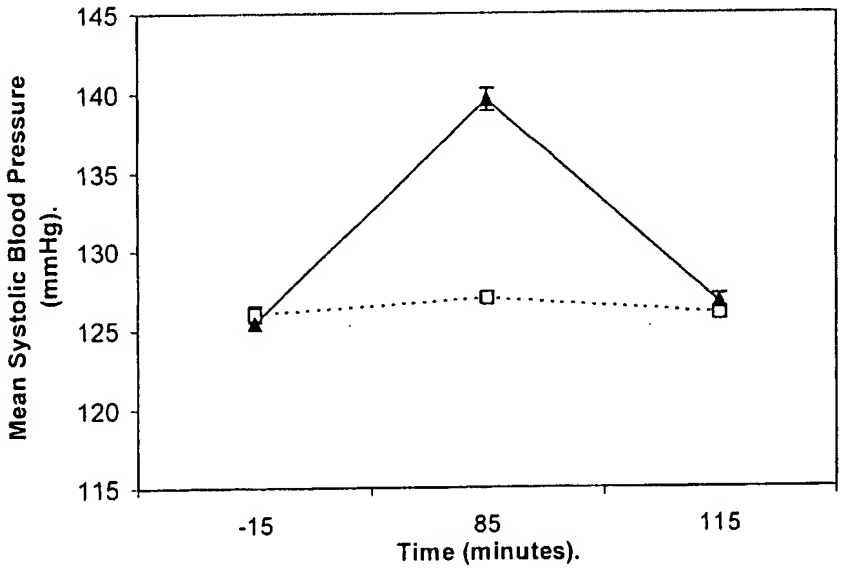
Blood samples were obtained from 32 healthy male and female subjects aged between 20-26 years before, during and after either watching an 83-minute horror film that none of the subjects had previously seen (The Texas Chainsaw Massacre, 1974) or by sitting quietly in a room (control group). Total differential cell counts, leukocyte activation as measured by the nitroblue tetrazolium test, heart rate and blood pressure measurements were taken at defined time points.

Figure 1. Haematological Variables: The effect of watching a horror film on subjects Open squares represents mean $\pm$ SEM from 16 control subjects Closed triangles represents mean $\pm$ SEM from subjects exposed to the horror film for all graphs.


Mean Leukocyte activation (%)
cells NBT positive.
45
40
35
30
25
20
15
10
5
0
-15
85
115
Time (minutes).


Mean Haemoglobin (g/l)
148
147
146
145
144
143
142
-15
85
115
Time (minutes)


90
80
70
60
50
40
30
20
10
0
Mean Heart Rate (bpm).
-15
85
115
Time (minutes).


145
140
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115
Mean Systolic Blood Pressure (mmHg).
-15
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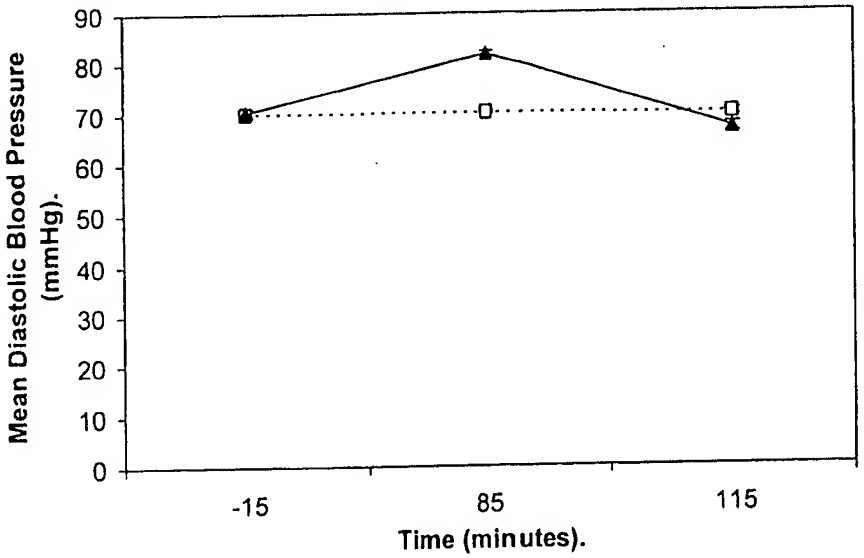
Time (minutes).



There were statistically significant increases in peripheral circulating leukocytes, the number of activated circulating leukocytes, haemoglobin concentration and haematocrit in response to the stressor (Mian *et al* 2003). These were accompanied by significant increases in heart rate, systolic and diastolic blood pressure. The study reveals the importance of the perception of stress. Watching a psychosocial stressful event that by definition has no objective effect on survival can thus affect immune reactivity.

The role of the psychobiological characteristics of the individual has been shown to be an important in the response to stress, for example Interleukin -1 (an indicator of stress) production is greater in right than in left-pawed mice (Neveu 2003).

The Mechanisms Involved

Watching a horror movie elicited psycho- physiological arousal which was comparable to Canon's fear –flight- fight- defence reaction, the so called 'stress response' which involves stimulation of the hypothalamus (Canon 1932; Folklaw 1982), a change in peripheral resistance (Brod 1972) and an increase in the release of stress hormones including catecholamines and cortisol (Seyle 1946) and increases in haematocrit and haemoglobin concentration (Maes *et al.*, 1998).

The increase in the numbers of circulating leukocytes during the horror film is comparable to that reported by others. Changes in circulating cell numbers reflects the cell trafficking between reservoir sites including the liver, lungs, spleen, bone marrow and peripheral blood (Cruse 1995). This process is modified by receptors (Hou 1996; Ley 1996) on both the endothelium (P-selectin; Intracellular Adhesion Molecule-1; Vascular cell Adhesion Molecule-1) and leukocytes (L-selectin; integrins and P-Selectin Glycoprotein Ligand-1 PSGL-1). Modification of the receptors on either the endothelial cells or leukocytes

can also dramatically alter the number of adherent (and thus the number of free flowing) leukocytes (Ley 1995; 1996).

PSGL-1 is constitutively expressed on all lymphocytes, monocytes, eosinophils and neutrophils Ley 1997. PSGL-1 has a glycosylation pattern enabling it to bind to endothelial P-selectin (Ley 1997). This interaction results in the margination of the leukocytes, which is the process by which leukocytes exit the central blood stream, and initiate mechanical contact with the endothelial cells (Cruse *et al.*, 1995).

The margination process is enhanced in vessels of a particular size by the aggregation of erythrocytes, which tend to occupy the centre of microvessels and thus promote margination (Firrell *et al.*, 1989). The increase in haematocrit and haemoglobin concentration observed in this and previous studies (Maes *et al.*, 1998) could thus selectively promote margination in some vessels. Previous studies have demonstrated that margination of leukocytes is not a uniform process, and occurs in particular sized vessels within the microcirculation (Mian *et al.*, 1993). In larger- sized vessels it is possible that the shear stress of flowing blood might be sufficient to dislodge marginated leukocytes, thus increasing the numbers of free-flowing leukocytes. The changes in shear stress likely to have been brought about by the increased haemoconcentration may also serve as a trigger mechanism for leukocyte activation (Schmid-Schonbeim *et al.*, 2001).

It has been recognised for some time that physiological stressors such as exercise induce leukocytosis from marginal pools (Shephard & Shek, 1996; Gleeson *et al.*, 1998). Current literature indicates that exposure to hostile conditions or other psychological stressors initiates the secretion of several hormones, including cortisol, catecholamines, prolactin, oxytocin and renin (Van de Kar & Blair, 1999; Toft *et al.*, 1994)), any of which could alter adhesion receptors on circulating leukocytes and thus contribute to their altered distribution. Stimuli such as adrenaline that disrupt this process (Iversen *et al.*, 1993) and increase the circulating numbers of leukocytes. Recent studies by Maes *et al.* (1999) have revealed that an increase in the levels of pro-inflammatory cytokines, such as interleukin-6 and tumour necrosis factor, result in the demargination of some leukocytes. It is thus possible that the stress -induced production of adrenaline and cytokines could orchestrate the increased numbers of leukocytes within the general circulation.

Non-physical stressors have now also been shown to influence the number and distribution of leukocytes in the blood. Kang *et al.*, (1996, 1997) and Dhabhar (1996) reported that the mental stress of academic examinations stimulated increases in the number and distribution of leukocytes. These changes were found to be both rapid and reversible.

Nitroblutetrazolium (NBT) reduction is a measure of cell activation which minimises in vitro manipulation of fresh blood, and reflects the in vivo condition of the cells. Recent studies have demonstrated that the NBT reduction assay is a reliable measure of the activation of neutrophils in whole blood (Takase *et al.*, 1999; Delano *et al.*, 1997). Wikstrom *et al.*, (1996) demonstrated a highly significant linear relationship between the % NBT –positive cells and chemiluminescence measurements of the same cell suspensions with and without chemical stimulation, supporting the idea that the NBT-test accurately measures oxidative metabolism. Thus the significant increase in the number of Nitroblue tetrazolium leukocytes after watching the horror film reflects an increase in the numbers of activated leukocytes. Activation of leukocytes has been reported by Kang *et al.*, (1996) who found that superoxidase production in neutrophils increased in those undertaking examinations.

The Pathophysiological Consequences of Stress by Proxy

In a broader context, the pathophysiological relevance of the changes observed need to be explored. If the human mind cannot discriminate observation of fictional stressful situations from personal psychological experience, then the results have implications for anyone witnessing a stressful event. Witnessing a stressful event could well be sufficient to alter the number and activation state of circulating leukocytes. Indeed the percentage of activated leukocytes remained high even though many of the other variables such as the number of circulating leukocytes and haematocrit and haemoglobin concentration had returned to basal (pre-stress) values. In this state of activation, leukocytes are primed and ready for action. If however the leukocytes actually release the contents of their granules, then there will be a period of time, a 'window of opportunity', in which they will not be able to respond to opportunistic infections, having already degranulated. Such leukocytes would be unable to respond to opportunistic infections thus rendering the host more susceptible to disease, as well as to potential tissue damage from a host of proteolytic enzymes and oxygen free radicals.

Unlike other cardiovascular and immune measurements, leukocyte activation was sustained for at least 20 minutes after the stress exposure, suggesting that this condition of the leukocytes is not rapidly reversible. The potential long-term effects of repeatedly watching such horror films remain unknown.

In an era where television and other electronic media rapidly transmit real and fictitious events into our homes, the effects of exposure to such stressors deserve evaluation. Witnessing a stressful event may have serious physiological consequences for the health of the observer. With sufficient exposure to psychological stress is it possible that an observer actually becomes a victim by proxy?

A Working Model of Neutrophil Behaviour in Response to Stress: the Key Players

Neutrophils are responsible for most of the ROS production seen in the blood samples (McLaren *et al.* 2003, Mian *et al.* 2003) A model of the interactions between neutrophils and other components of the stress response is outlined in Figure 3 and discussed below.

Plasma Factors

Stress causes the release of stress hormones including cortisol and adrenaline. A host of stress-related endocrine and non-endocrine plasma borne factors could potentially modify the sensitivity of circulating neutrophils to PMA and control their level of activation. For example, neutrophil behaviour, including ROS, is modified by both cortisol (e.g. Kurogi & Iida, 2002) and catecholamines (e.g. Bergmann & Sautner, 2002; Benschop 1999).

Cytokines

Non-endocrine plasma-borne factors could also potentially control the response of neutrophils in response to stress. In particular the cytokines IL- 1 & IL- 6, which are known to be released from activated endothelial cells, are known to affect neutrophil accumulation and activation(e.g. Joseph *et al.*, 1992). Also, IL- 8 is an important chemo-attractant for neutrophils and can also play a role in the production of ROS (McPhail & Harvath, 1993), and thus may play an important role in the recruitment and activation of neutrophils during stress.

Glutamine

Other factors that are important for neutrophil function, such as glutamine (Furukawa *et al.*, 1997; 2000; Pithon-Curi *et al.*, 2002) and glucose (Furukawa *et al.*, 2000), could also affect the response of neutrophils to stress. In the case of glutamine, there is evidence to support the notion that glutamine depletion is related to immunosuppression in athletes (Castell & Newsholme 2001; Castell 2002).

Neutrophil-Endothelial Cell Interactions

The adhesion of neutrophils to the endothelium is another important component of neutrophil activation during stress (Jean *et al.*, 1998; Figure 3) and modification of the receptors on either the endothelial cells or neutrophils can dramatically alter the number of adherent (and thus the number of free flowing) neutrophils and the distribution of leukocyte subsets (Ley, 1996; Maes 1999). Endothelial and neutrophil derived adhesion molecules serve important roles in properly orienting neutrophils temporally and spatially for activation along the endothelium (Park & Lucchesi, 1999;). Important regulators in this process are TNFa, and ICAM- 1. TNFa upregulates ICAM- 1, which leads to increased neutrophil-endothelium interaction (Menger *et al.*, 1999). The selectin family of adhesion molecules, which includes E- and L- selectin, mediate the first contact of neutrophils with the endothelium. (Ley, 1996). E- selectin is expressed on the surface of endothelial cells and L- selectin is expressed on the surface of neutrophils. L- selectin can also influence the production of reactive oxygen species (Nagahata *et al.*, 2000). Furthermore, changes in shear stress, such as those brought about by stress related increases in red blood cell numbers, can modify neutrophil adhesion behaviour (Sheikh *et al.*, 2003).

In summary stress-induced changes in leukocyte activation reflect changes occurring in the local environment.

THE COST OF IMMUNOSUPPRESSION: RESOURCE LIMITATION OR SELF PROTECTION?

The adaptive significance of immunosuppression is hypothesised to be related to either resource limitation or for self-protection through the avoidance of immunopathology (Råberg *et al.* 1998). The resource limitation hypothesis predicts that immunosuppression occurs so that energy and nutrients can be temporarily shifted away from the immune system and diverted into cells and tissues that are directly required to cope with the stressor. In this model there is a resource-based trade-off between the immune system and the costly behaviours associated with coping with the stressor. The extent of immunosuppression is thus predicted to be related to the intensity of the stressor and the condition of the animal.

The self-protection hypothesis predicts that the immune system is suppressed in order to protect the organism from 'hyperstimulation' of the immune system (Råberg *et al.* 1998). Some components of the immune system are activated by stress: for example some leukocytes (particularly neutrophils) can become activated and release oxygen free radicals (Mian *et al.* 2003; Montes *et al.* 2003; Montes *et al.* in press). Oxygen free radicals are potentially damaging to the host organism, costly to produce, and damage the activated leukocyte in the process of their release (Halliwell & Gutteridge 2000. Although the two hypotheses to explain immunosuppression are not mutually exclusive, they do make different predictions regarding the nature of stress-induced immunosupression.

There is considerable evidence that investment in the immune system is, in non-stress situations, dependent upon an animal's condition and nutritional status. For example, male Belding's ground squirrels (*Spermophilus beldingi*) that received a food supplement had a leukocyte count three times greater then control males (Bachman 2003). Furthermore, glutamine, an important fuel for immune cells (Castell 2002; Castell & Newsholme 2001), is known to enhance PMA- induced oxygen free radical production in neutrophils (Pithon-Curi et al. 2002). Low plasma glutamine is associated with a decrease in the functional ability of immune cells (Castell & Newsholme 2001) and in humans glutamine levels in the blood are decreased after prolonged, exhaustive exercise (Castell 2002).

To test the predictions of the two hypotheses. We subjected wild small mammals (woodmice Apodemus sylvaticus and bank voles Clethrionomys glareolus) to one of two treatments: handling (stressor) and non-handling(McLaren *et al.* submitted). We then measured the ability of animals' circulating leukocytes to produce oxygen free radicals in response to PMA challenge: the leukocyte coping capacity (LCC). Handling suppressed the LCC response, both in magnitude and variability. Heavier non-handled animals had a greater peak LCC, but this was not the case in handled animals, contrary to the predictions of the resource hypothesis. The self-protection hypothesis also failed to predict the observed variation we found in LCC in handled animals.

During an immune response, leukocytes are activated to produce oxygen free radicals during a stressful event, and this most likely occurs to prepare the body for physical damage and perhaps infiltration by bacteria (Mian 2003). In evolutionary terms it is tempting to speculate that stress would generally be linked to a fight or aggression that may lead to physical injury, thus the release of oxygen free radicals may be a 'pre-emptive strike' anticipating injuries and associated bacterial infection.

Once sufficient oxygen free radicals have been released it is possible that other mechanisms ensure that further production is suppressed to protect the body from physical damage. This model needs further investigation, in particular studies relating plasma glutamine levels to body weight in mammals and *in vitro* studies examining patterns of stress-related oxygen free radical production in relation to glutamine availability (Castell & Newsholme 2001; Pithon-Curi *et al.* 2002).

It is clear that the self-protection and resource hypotheses are not mutually exclusive, and both may play a part in the immunosuppression we observed in handled mice and voles. More generally, we hypothesize that the level and nature of stress-induced immunosuppression will vary between different components of the immune system depending upon: (1) the potential for the component of the immune response to cause harm to host tissues; (2) the energetic cost of the immune component; (3) the intensity, frequency and duration of the stressor; and (4) the condition of the animal. This hypothesis predicts that immune responses that are unlikely to damage the host may be more likely to be suppressed to save resources, whereas potentially damaging responses are suppressed to protect the host.

A Working Model of Neutrophil Behaviour in Response to Stress

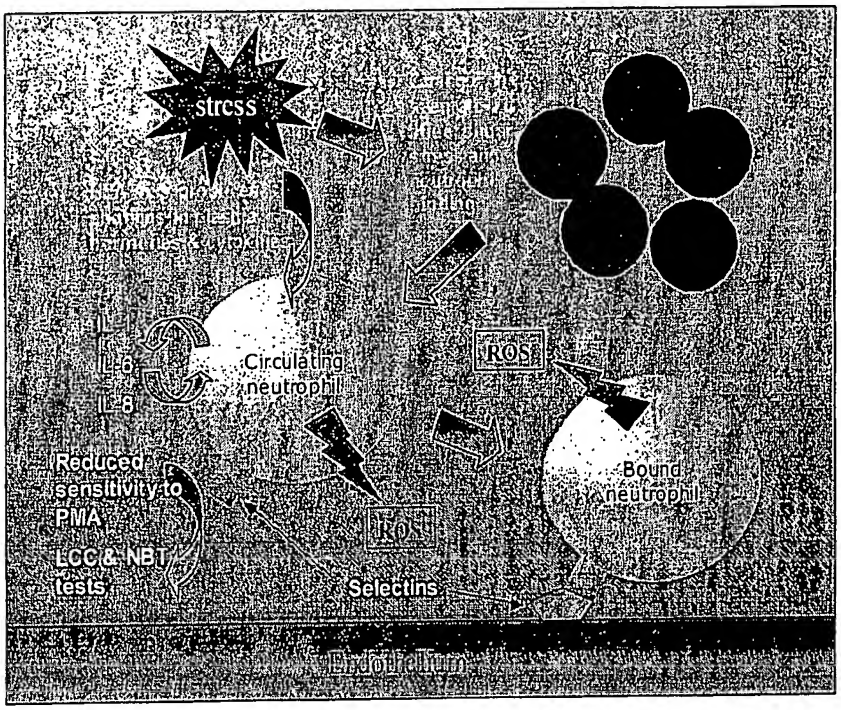


Figure 3.

This diagram represents our working model of the interaction between stress and neutrophil behaviour. Stress brings about a shift in the behaviour of neutrophils, inducing the

release of reactive oxygen species (ROS), and initiating a sequence of events that leads to neutrophils binding to the blood vessel endothelium, and (not shown) rolling along the endothelium and migrating into the tissues. Cytokines (including IL-1, IL-6, and IL-8) and stress hormones are likely to play a role in initiating and maintaining this behaviour, as is shear stress. This shift in responsiveness is limited to a subpopulation of neutrophils: those remaining in the circulation have a reduced sensitivity to PMA (see text for details), and this can be used as a measure of stress.

A New Look at Stress-Induced Production of Oxygen Free Radicals: Therapeutic Possibilities

Phagocytic leukocytes play a pivotal role in the innate immune response against bacteria, fungi , foreign particles and stress induced immuno-supression (Ellard *et al.* 2001, Mian *et a.l* 2003, McLaren *et al.* 2003). On the surface of phagocytic leukocytes lies NADPH oxidase, multisubunit enzyme that can assemble which when assembled can 'shoot' highly reactive oxygen species (ROS) through the membrane. NADPH oxidases are tightly controlled and thus generally do not blast highly reactive superoxide anions into healthy tissues. It has recently been shown that once 'superoxide shooting' commences, the leukocyte initiates a highly coordinated sequence of events which include fusion and release of several types of granules and activation of antimicrobial enzymes (Bokoch 2002).

The role of ROS is not thus just that of a reactive oxygen free radical, but may be a signal for subsequent alteration of electrons, movement of ions and ultimately release of granular contents (Bokoch 2002). Thus an alteration of stress-induced ROS (Figure 4 from Bokoch 2002) may signal a sequence of more sinister alterations of immune function.

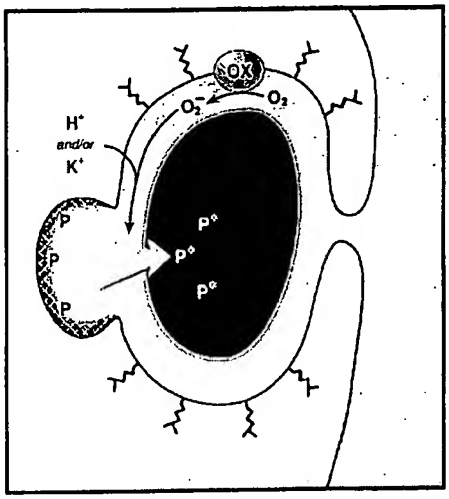


Figure 4.

The single electron reduction of molecular oxygen to form superoxide anion by the phagocyte NADPH oxidase (OX), stimulated by bacterial uptake (and possibly by substances

e.g. adhesion molecules or cytokines released by stress), results in the transfer of electrons into the enclosed phagocytic vesicle. Dismutation of the superoxide generates OH^-, and the accumulating negative charge must be compensated by the influx of H^+ and/or K^+. The hypertonicity resulting from K^+ transport promotes the release of inactive cationic granule proteases (P) bound to an anionic sulfated proteoglycan matrix (cross-hatching). The released and active proteases (P^*) encounter the bacterium under optimal pH conditions within the phagocytic vesicle and degrade it. Cytoskeletal elements associated with the phagocytic vesicle (wavy lines) indirectly affect the killing process by modulating vesicular volume. pH and movement of ions may well be affected by gas signalling molecules. We speculate that this process may be intiated by stress. Pharmacological intervention with any of the processes discussed may modulate the production of ROS.

Figure reprinted from Bokoch (2002), with permission from GM Bokoch and the Nature Publishing Group.

ROS Production in Disease States

Understanding the relationships between stress and ROS production is also important for disease studies. ROS have been implicated in a wide variety of autoimmune diseases such as rheumatoid arthritis (Halliwel *et al.*, 1992) inflammatory bowel disease (Kruidenhier & Verspaget 2002) and systemic lupus erythematosus (SLE) (Ames *et al.*, 1999) and psoriasis (Pereira *et al.*, 1999). Stress related psoriasis has been documented (Alabadie *et al.*, 1994). There is evidence that the occurrence of psoriasis is related to increased ROS production and decreased antioxidant capacity (Baz *et al.*, 2003). In an interesting experiment Kabat- Zinn *et al.*, (1998) demonstrated that stress reduction by meditation (in conjunction with ultraviolet light therapy) increased the rate at which psoriasis cleared in patients. ROS have been linked to such a variety of diseases because they of their potential for causing wide-ranging tissue damage. ROS can damage DNA and membranes and the oxidation products can induce protein damage, apoptosis, and the release of pro-inflammatory cytokines (Briganti & Picardo 2003), leading to serious tissue damage if antioxidant capacity is insufficient.

In patients with SLE the severity of the disease is known to be related to daily psychological stress (Pawlak *et al.*, 2003), and given that oxidative stress is a known factor in this disease (Ames *et al.*, 1999), the relationships between psychological stress, ROS production and disease onset and severity would be worth exploring further.

Concluding Comments

All mammals are subjected to psychological stress at some point in their lives. Whether man or mouse the nature, duration and intensity of the stress can result in a common endpoint : immunosupression and the release of reactive oxygen species from circulating leukocytes. Even vicarious, fictitious stress is sufficient to orchestrate what appears to be a basic instinctive response – the preparation for ensuing bacterial invasions. If the stress is of sufficient magnitude we appear to be hard wired to produce a defined physiological response. We prepare ourselves for action or injury. In anticipation, leukocytes prepare for battle and release their arsenal of weapons. The response is regulated. If however the stress continues,

then this can have devastating consequences on the host (Irie 2003). Long term immunosupression can result in an increased vulnerability to opportunistic infections. Perhaps the ancient Egyptians were aware of the power of stress when they wrote curses on the tombs of pharaohs to deter thieves

> 'Moreover, as for him who shall destroy this inscription: He shall not reach his home. He shall not embrace his children. He shall not see success."
>
> (Budge 2001)

Such words would doubtless terrify a superstitious, and already anxious, tomb- raider. The resulting psychological stress, together with scattered invisible moulds scattered on the bodies of the mummies, which could remain dormant for thousands of years, could be a fatal combination. Believing in the curse could indeed have been a self-fulfilling prophesy. Unwittingly or otherwise than ancient Egyptians may have designed the first effective psycho-immunodeterrents.

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The effect of stress on the immune response of Atlantic salmon (*Salmo salar* L.) fed diets containing different amounts of vitamin C

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ABSTRACT

An experiment was performed to determine the effects of dietary vitamin C levels and stress on immunological parameters in Atlantic salmon (*Salmo salar* L.). Atlantic salmon parr were maintained on diets containing 0.082 (low), 0.44 (normal) and 3.17 (high) g vitamin C/kg dry diet. After 23 weeks, tissue levels were found to reflect dietary input. Groups of fish were removed and subjected to a 2 h confinement stress prior to sacrifice and isolation of plasma and head kidney leucocytes. Leucocyte respiratory burst activity ($P<0.01$) and bactericidal activity ($P<0.05$) were both found to be significantly reduced by stress, but were unaffected by vitamin C status. Leucocyte migration was unaffected by stress or vitamin C status. Plasma bactericidal activity was also unaffected by vitamin C status but was significantly enhanced by stress ($P<0.05$). Production of specific antibody following immunisation with *Aeromonas salmonicida* was found to be significantly reduced ($P<0.01$) by stress, and there were significantly greater levels of specific antibody ($P<0.01$) in fish fed the low vitamin C diet compared with fish fed high levels of vitamin C.

INTRODUCTION

The physiological response of fish exposed to a stressor can be described as being made up of primary, secondary and tertiary components (Mazeaud et al., 1977; Wedemeyer and McLeay, 1981). The primary effect involves increased activity of the hypothalamic–pituitary–interrenal (HPI) axis, resulting in increased levels of catecholamine and glucocorticoid hormones which induce a wide variety of secondary effects including metabolic, haematological, hydromineral and structural changes (reviewed by Barton and Iwama, 1991). One such secondary effect is the elevation of plasma glucose during the stress response in salmonids, providing a more stable if less sensitive method of differentiating between control and stressed fish than the measure-

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ment of cortisol itself (Pickering et al., 1982). Such secondary changes ultimately result in tertiary effects, i.e. those which are manifest as gross changes in physiological performance at the whole organism level.

One tertiary effect of particular relevance to the aquaculture industry is the decrease in disease resistance of stock, generally accepted as being attributable to the immunosuppressive effect of elevated levels of cortisol (reviewed by Pickering, 1989; Barton and Iwama, 1991). Increased levels of circulating cortisol affect the teleost immune system at many levels. The number of circulating leucocytes is reduced (Pickering and Pottinger, 1987) and the composition of the leucocyte population is affected, with lymphocytopenia and neutrophilia generally being apparent (Pickering et al., 1982; Ellsaesser and Clem, 1987). There are numerous examples of the effects of cortisol on non-specific defence mechanisms. Examples include inhibition of *in vitro* respiratory burst activity (Stave and Robertson, 1985; Angelidis et al., 1987) and *in vivo* phagocytic activity (Ainsworth et al., 1991). Leucocyte migration has also been shown to be reduced following injection with cortisol (MacArthur and Fletcher, 1985) and non-specific cytotoxic cell activity has been shown to be suppressed in socially subordinate fish (Ghoneum et al., 1988). Cortisol also effects specific immune responses. It has been shown that mitogen responses and antibody production by lymphocytes from stressed fish are significantly depressed (Ellsaesser and Clem, 1986; Kaattari and Tripp, 1987). The effects of reduced lymphocyte function are also apparent in vaccinated fish which have similar mortality rates to their unvaccinated counterparts on post-stress challenge (Houghton and Matthews, 1986).

Different strategies have been proposed as being potentially beneficial for reducing the physiological effects of stress in farmed fish, such as feeding elevated levels of vitamin C (Jaffa, 1989; Hardie et al., 1991), for which precedents exist in various domestic animals, notably chickens (Brake et al., 1992; Pardue and Williams, 1992). Vitamin C, which also has beneficial effects on the teleost immune system (reviewed by Blazer, 1992), may act as a brake on steroidogenesis through peroxidation of unsaturated lipids, thereby preventing their conversion into cholesters which are important components of cortisol (Kitabchi, 1967). Early experiments with salmonids appeared to confirm that steroidogenesis was inhibited by high levels of vitamin C in the interrenal tissue (Wedemeyer, 1969, 1972). However, recent results have indicated that cortisol levels post stress may be independent of the vitamin C status of the fish (Dabrowska et al., 1991).

While antioxidant vitamins such as C and E appear to increase disease resistance at the tertiary level, it has proved difficult to find many significant effects on the immune system at the secondary level, with the most marked effect being an elevation of serum complement levels (Li and Lovell, 1985; Hardie et al., 1991). Effects may be more apparent at the secondary level at times when the potential demand for vitamin C is increased i.e. under stress-

ful conditions. In this study, Atlantic salmon parr (*Salmo salar* L.) maintained on low, normal and high vitamin C diets were subjected to a 2 h confinement stress, in order to investigate whether high levels of vitamin C can ameliorate the stress-induced down regulation of the immune system. The effect of stress on a representative range of humoral and cell-mediated immune parameters should ascertain whether high vitamin C diets provide a practical means of minimising stress-induced immunosuppression in aquaculture, and provide more information on the direct effects of vitamin C on the salmonid immune system.

MATERIALS AND METHODS

Maintenance of salmon

Atlantic salmon parr (mean weight 15 g) were maintained at the Scottish Office Agriculture and Fisheries Department's (SOAFD) Marine Laboratory in Aberdeen. The fish were kept in a semi-recirculating culture system incorporating both chemical and biological filters, comprising six 420-l capacity circular tanks containing 1/3 strength sea water supplied at 10 l/min at ambient temperature (range 8–12°C), and a light regime of 10 h light/14 h dark. Four hundred and twenty parr were equally distributed between the tanks. The parr were maintained on a fish meal based diet (after Hardie et al., 1991) that conformed to National Research Council (NRC) specifications excepting vitamin C levels. The basal diet contained 0.082 g vitamin C/kg dry diet (low) and this was further supplemented to give final concentrations of 0.44 g/kg (normal) and 3.17 g/kg (high) as determined by analysis according to the method of McGown et al. (1982), with a lower limit of sensitivity of 0.2 μg/ml. Paired tanks of parr were fed with each test diet at a rate of 2% body weight/day 7 days a week for 23 weeks. The total weight of each group was determined once a month, and feeding rates adjusted accordingly. After 23 weeks, specific growth rates (SGR; % body weight day^{-1}) for each group were calculated using the equation:

$$SGR = 100(\log_n W_f - \log_n W_o)/t$$

where W_o and W_f were the initial and final group weights respectively after t days (Ricker, 1979).

After 23 weeks, five fish were sacrificed from each tank for the assessment of endogenous liver and kidney vitamin C levels.

Experimental procedure

Experiments were carried out each week, commencing in week 23. On the first morning of each experiment, five control fish were sampled at random

from the deficient ration group, killed by a blow to the head and bled via the caudal vein with a heparinised syringe within 2.5 min. Differential leucocyte counts were made on duplicate blood smears from each fish. Slides were air-dried, fixed in methanol and stained with Giemsa. Excluding thrombocytes, a total of 200 lymphocytes, neutrophils and monocytes were counted on each slide, in oil immersion fields, in strips running the length of each film. The remaining blood was centrifuged at $10000 \times g$ for 5 min and the plasma removed for glucose analysis on the same day. The remaining plasma was stored at $-70°C$ for future analysis. After bleeding, each control fish was dissected. The head kidney was removed and placed on ice in Leibovitz medium (L15; Gibco) containing 2% foetal calf serum (FCS) and 0.2% heparin. Livers were also removed and stored at $-70°C$ for subsequent vitamin C analysis. Five further fish were sampled at the same time to investigate the effects of stress, and these were placed in an opaque covered plastic bucket (base diameter 30 cm) containing 5 l of aerated tank water. These fish were transported for 15 min and, after a total of 2 h confinement, they were killed and processed as described for control fish. Normal and high vitamin C status fish were sampled on successive days.

Quantification of stress

Plasma glucose was determined using a commercial kit (Miles). This kit works on the principle that glucose is converted by glucose oxidase into gluconic acid and hydrogen peroxide which, in the presence of peroxidase, oxidises the chromagen (4-aminophenazone/phenol) to a red coloured compound. Briefly, 20 μl of fresh plasma was added to 2.5 ml of supplied working solution and incubated at 37°C for 15 min. The optical density (O.D.) was then determined at 505 nm against a working solution blank. The absorbance of the sample/absorbance of a 5.5 mmol l^{-1} glucose standard $\times 100 =$ plasma glucose in mg/dl.

Respiratory burst

Respiratory burst activity of phagocytes within a suspension of kidney leucocytes was quantified by measuring the reduction of ferricytochrome C by extracellular O_2^- radicals produced by membrane stimulation with phorbol 12-myristate 13-acetate (PMA; Sigma). Head kidney leucocytes were isolated as described previously for rainbow trout (Chung and Secombes, 1988). Briefly, the head kidney from each fish was pushed through a 100 μm nylon gauze to produce a cell suspension which was layered onto 51% Percoll (Sigma). Following centrifugation at $400 \times g$ for 30 min, the purified leucocytes were removed, washed twice in L15 medium containing 2% FCS and 0.2% heparin, and adjusted to 10^6 viable cells/ml. Two 1-ml aliquots per fish were centrifuged at $5000 \times g$ for 10 s to avoid damaging the cells. The supernatants were discarded and one tube was supplemented with ferricytochrome

C (2 mg/ml in phenol red free Hanks balanced salt solution pH 7.4; HBSS) containing PMA at 1 μg/ml. The second tube was supplemented with ferricytochrome C (Sigma) containing PMA and superoxide dismutase (SOD) at 300 U/ml to confirm the specificity of the reaction. Cells were resuspended by vortexing, and incubated at room temperature for 15 min with regular mixing. The reaction was then terminated by centrifugation at 5000×*g* for 1 min. One hundred-μl samples were removed in triplicate from each pair of tubes and the O.D. measured at 550 nm against a ferricytochrome C blank in a multiscan spectrophotometer (MDC Thermomax). Readings were converted to nmoles O_2^- by subtracting the O.D. of the PMA/SOD treated supernatant from that treated with PMA alone for each fish, and converting O.D. to nmoles O_2^- by multiplying by 15.87 (Pick, 1986). Final results were expressed as nmoles O_2^- produced per 10^5 kidney leucocytes.

Cellular bactericidal activity

Killing of *Aeromonas salmonicida* (strain MT004) by a suspension of kidney leucocytes was investigated using a modified assay based on that of Graham et al. (1988). Leucocytes were isolated and purified as described for the respiratory burst. Cells from each fish were resuspended to a final concentration of 4×10^7/ml in L15 medium containing 5% FCS (screened for bactericidal activity) and 50 μl of suspension was added (one column per fish) to a 96-well microtitre plate. Fifty μl of L15 was added to additional wells to act as the plate blank, and also to control for contamination. Twenty μl of a bacterial suspension (5×10^3/ml in 3% tryptic soy broth; TSB) was added to each of the top 4 wells for each fish (T5 wells), and 20 μl of 3% TSB was added to the blank. The plate was then shaken and incubated at 18°C for 5 h. Twenty μl of the same bacterial suspension (also incubated at 18°C for 5 h) was then added to the lower 4 wells for each fish (T0 wells). The plate was then shaken and 30 μl of lysis buffer (5% Tween 20 in distilled water) was added to all wells to lyse the leucocytes and stop their bactericidal activity. One hundred μl of 5% TSB was then added to all wells to support bacterial growth, and the plate incubated at 18°C for 16 h. After 16 h the plate was shaken vigorously to overcome interference from membrane fragments and 100 μl of bacterial suspension from each well was transferred to another microtiter plate. Ten μl of 3-[4,dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) at 5 mg/ml in distilled water was then added to all wells and the plate was incubated in the dark for 15 min before measuring the O.D. at 600 nm. Bactericidal activity of leucocytes was calculated by subtracting the T0 group mean from the T5 group mean, with results expressed as % bacteria killed.

Leucocyte migration

The migratory response of salmon kidney leucocytes to fresh serum was investigated using a microchemotaxis chamber (Neuroprobe, MD, USA). A

leucocyte suspension was prepared as described earlier and layered onto a 34%/51% Percoll gradient. Following centrifugation, the phagocyte-enriched fraction at the interface was removed and washed twice in HBSS pH 7.4 containing phenol red. Cells from each fish were then resuspended to a final concentration of 5×10^6/ml in this medium. A 1:50 solution of fresh salmon serum in HBSS was prepared and pipetted in triplicate into the lower wells of the chemotaxis chamber. Triplicate wells of HBSS alone served as controls. A 3 μm pore size polyvinylpyrrolidone-free polycarbonate filter (Millipore, Bedford, MA, USA) was then placed over the bottom wells followed by a silicon rubber gasket and finally the upper wells. Forty-three μl of each leucocyte suspension was then added in triplicate to both the control and chemoattractant-treated wells. The chamber was then incubated in the dark at 18°C in a moist chamber for 90 min. After incubation the filter was removed and the non-migrated leucocytes on the upper surface of the filter were scraped off by running the filter 4 times in a unidirectional manner along a rubber blade. Filters were then air dried, stained in Giemsa (10% in Gurr buffer pH 6.8; BDH) for 30 min, and mounted on microscope slides. For each fish, numbers of migrated cells were estimated by counting the number present in 5×1 mm^2 fields of view in both control and chemoattractant-treated areas of the filter at ×400 magnification using an eyepiece graticule. Results were expressed as the number of cells migrating above background levels per mm^2 of filter.

Plasma bactericidal activity

Seventy-five μl of a suspension of *A. salmonicida* (10^8/ml in 3% TSB) was added to 25 μl of plasma in triplicate in a 96-well microtiter plate. Bacteria were also added to wells containing 25 μl of 3% TSB as controls. The plate was shaken and incubated at 18°C for 3 h, shaking hourly. The plate was then centrifuged at $150 \times g$ for 10 min and the supernatants removed. MTT (5 mg/ml H_2O) was diluted 10-fold in 3% TSB and 100 μl was added to all wells. The plate was then incubated in the dark at 18°C for 15 min and read in a multiscan spectrophotometer (MDC Thermomax) at 600 nm. Killed bacteria were quantified by subtracting the O.D. measured for each test plasma sample from the mean control O.D. Results were expressed as the % bacteria killed.

Specific antibody response

After 23 weeks on their respective diets, ten fish from each ration group were subjected to confinement stress, anaesthetised in benzocaine (25 μg/ml) and fin clipped for identification purposes immediately prior to immunisation with formalin-killed *A. salmonicida* (5 mg/ml in PBS) by intraperitoneal injection. Five control fish were similarly immunised. Ten further fish from each vitamin C status group were bled via the caudal vein at the time of immunisation to provide a basal antibody titre for each group. Five stressed

and unstressed immunised fish from each vitamin C status group were subsequently bled 29, 43 and 64 days post immunisation. Antibody titres were measured by adding 50 μl of formalin-killed *A. salmonicida* (10^9/ml) to 50-μl aliquots of fresh serum serially diluted (2-fold) in a 96-well microtiter plate. Following overnight incubation at 4°C, the last dilution of serum giving a positive reaction was taken as the titre. After bleeding on day 64, all fish were killed and the livers removed for vitamin C analysis.

Statistical methods

Vitamin effects, stress effects and vitamin/stress interactions were investigated by two-way analysis of variance followed by Tukey's multiple range test where appropriate.

RESULTS

Growth performance and haematological parameters

No differences were observed in the specific growth rates among groups of fish fed the low (0.24% body weight day^{-1}), normal (0.21% body weight

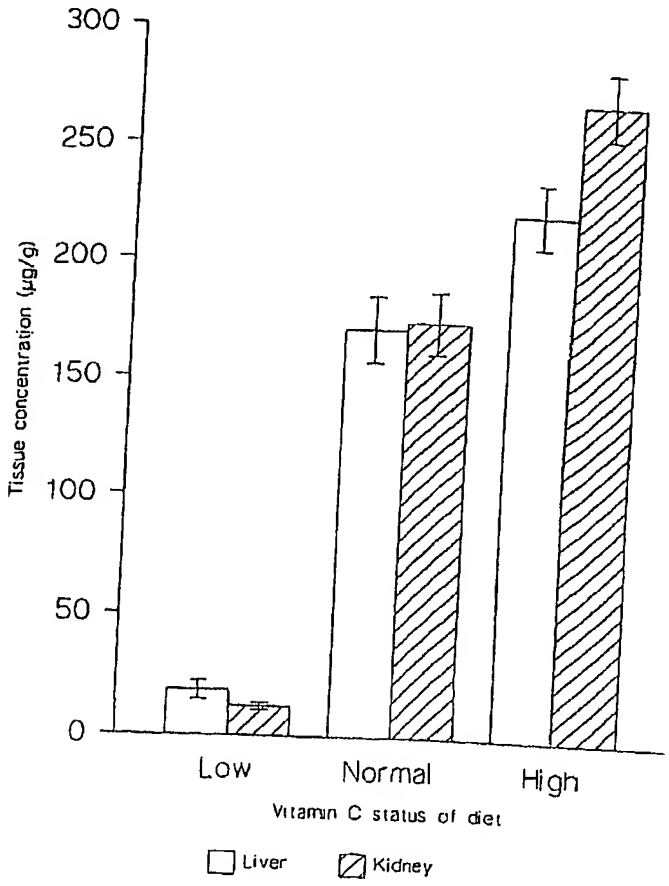

Fig. 1. Endogenous vitamin C levels in liver and kidney of salmon after feeding three levels of vitamin C for 23 weeks. Data are expressed as means of 10 fish ± standard error.

TABLE 1

Control and 2 h post-stress values of liver vitamin C (μg/g) and plasma glucose (mg/dl) from experiments involving salmon fed three different levels of vitamin C. Results are means ± standard error

Experiment	Parameter	Low		Normal		High	
		Control	Stressed	Control	Stressed	Control	Stressed
Leucocyte respiratory burst ($n=5$)	Liver vit C	14.4 ±0.7	17.1 ±4.8	117.6 ±6.8	112.1 ±3.9	257.4 ±17.3	251.2 ±21.2
	Plasma glucose	91.3 ±3.8	189.0 ±10.9	71.7 ±5.3	199.0 ±11.1	88.2 ±3.8	194.6 ±7.4
Leucocyte bactericidal activity ($n=5$)	Liver vit C	10.2 ±2.8	10.7 ±2.6	100.2 ±11.1	110.7 ±10.6	251.9 ±9.1	232.2 ±9.1
	Plasma glucose	113.8 ±3.1	204.5 ±9.7	121.7 ±4.1	220.5 ±11.5	108.4 ±6.2	206.0 ±7.4
Leucocyte migration ($n=4$)	Liver vit C	8.8 ±1.8	11.9 ±2.9	133.0 ±7.4	128.2 ±7.6	225.4 ±10.4	218.2 ±6.8
	Plasma glucose	88.3 ±3.8	198.8 ±3.8	82.4 ±3.0	189.7 ±4.3	82.0 ±3.0	191.4 ±5.6
Plasma bactericidal activity ($n=5$)	Liver vit C	8.8 ±2.2	8.6 ±1.1	119.4 ±6.9	124.7 ±7.3	215.9 ±6.8	220.5 ±6.8
	Plasma glucose	90.7 ±3.9	193.8 ±3.9	85.6 ±2.7	181.7 ±2.7	90.9 ±3.1	187.9 ±3.3

TABLE 2

Control and 2 h post-stress differential leucocyte counts determined from salmon fed three different levels of vitamin C. Data are expressed as means of five fish ± standard error

	Low		Normal		High	
	Control	Stressed	Control	Stressed	Control	Stressed
Lymphocytes (%)	89.6±2.0	92.0±1.0	85.2±2.4	88.4±2.2	87.2±1.6	92.2±0.6
Neutrophils and monocytes (%)	10.4±2.0	8.0±1.0	14.8±5.4	11.6±2.2	12.8±1.6	7.8±1.3

day^{-1}) and high (0.23% body weight day^{-1}) vitamin C diets. All three groups continued to feed normally throughout the experiment, and there was no sign of scoliosis or lordosis in the group fed the low vitamin C diet. Liver and kidney levels sampled at week 23 reflected dietary input (Fig. 1), with signif-

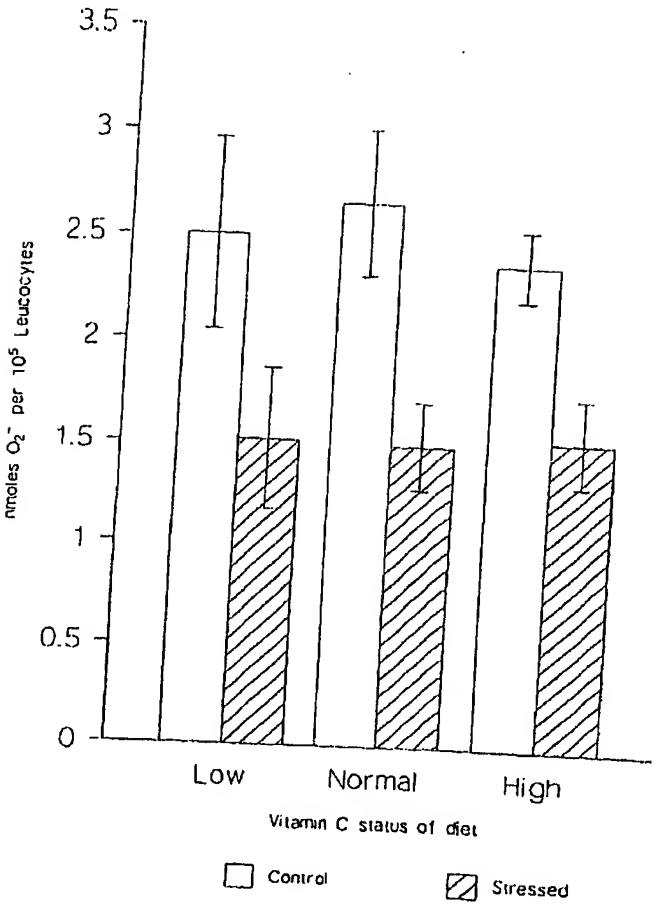

Fig. 2. Respiratory burst activity of head kidney leucocytes isolated from control and 2 h post-stress salmon fed three different levels of vitamin C. Data are expressed as means of 5 fish ± standard error.

icantly more vitamin C being present in these tissues in the high dose group compared with the normal group ($P<0.01$), and the normal group compared with the low dose group ($P<0.001$). Distribution of vitamin C between the liver and kidney in the deficient and normal groups was similar (Fig. 1), but significantly higher levels ($P<0.05$) were found in the kidney compared with the liver of the group fed the high vitamin C diet. These observations may indicate that liver levels were becoming saturated in the fish fed the high vitamin C diet.

Liver vitamin C levels were found to be unaffected by 2 h confinement stress (Table 1) and a pilot study showed that liver and kidney vitamin C levels were also unaffected by a 2 h confinement stress, even when applied on a daily basis for several consecutive days (unpublished data). The plasma glucose concentration in the control fish was found to be similar across the range of diets in all experiments (Table 1), indicating that there was no difference in the underlying stress status of the three vitamin C status groups. Similarly, the magnitude of the hyperglycaemic response to confinement stress

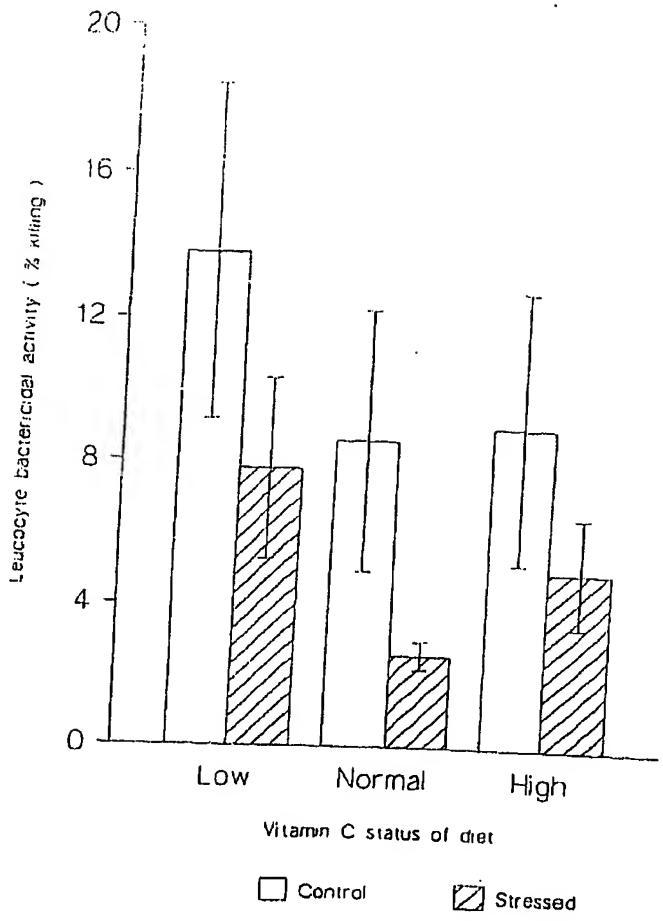

Fig. 3. Bactericidal activity of head kidney derived leucocytes isolated from control and 2 h post-stress salmon fed three different levels of vitamin C. Data are expressed as means of 5 fish ± standard error.

(an approximately 2-fold increase in plasma glucose levels) was found to be independent of the vitamin C status of the fish, providing evidence that there may be no difference in the susceptibility of the three vitamin C status groups to stress.

Blood samples taken 2 h after the onset of confinement stress showed that there was a significant change in the composition of the leucocyte population. The % lymphocytes was elevated and the % neutrophils/monocytes suppressed in the stressed fish compared with the control groups ($P<0.05$) (Table 2).

Non-specific cell-mediated immune responses

Phagocytes isolated 2 h after the onset of confinement stress showed a significantly reduced ability to undergo a respiratory burst ($P<0.01$) compared with control fish (Fig. 2), with a mean reduction in O_2^- production of approximately 40% across the three vitamin C status groups. However, the respiratory burst activity was found to function independently of vitamin C sta-

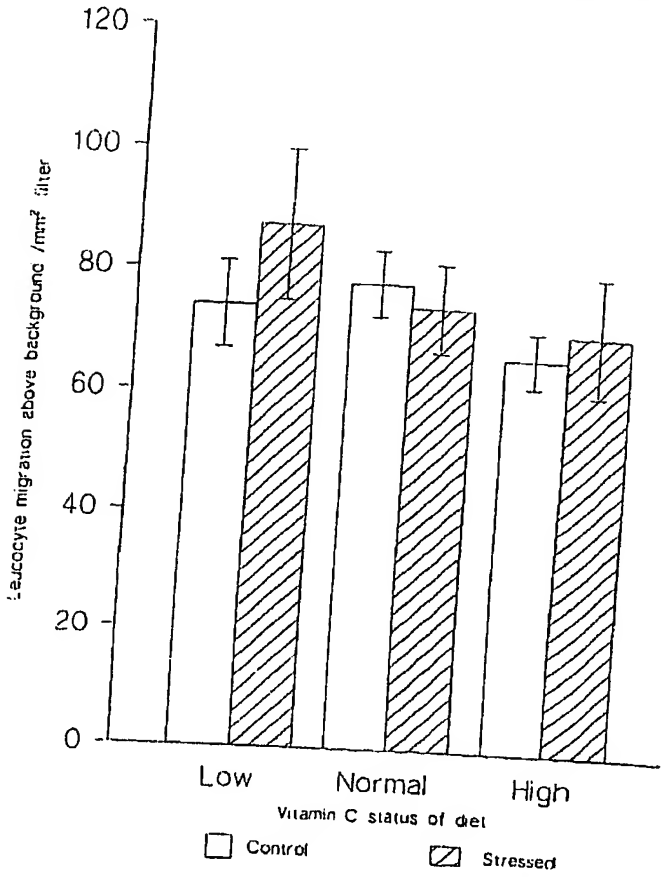

Fig. 4. Migration of head kidney derived leucocytes isolated from control and 2 h post-stress salmon fed three different levels of vitamin C. Data are expressed as means of 4 fish ± standard error.

tus and there was no evidence of any vitamin C-mediated amelioration of this immunosuppression. Leucocyte bactericidal activity was also significantly reduced by stress ($P<0.05$) (Fig. 3), with a mean reduction in bactericidal activity of around 50% across the three vitamin C status groups. Once again, bactericidal activity was found to be independent of vitamin C status and there was no evidence of any vitamin C-mediated amelioration of this immunosuppression. Migration of HK-derived leucocytes to a serum stimulus was found to be unaffected by vitamin C status or stress (Fig. 4).

Non-specific humoral immune response

Plasma samples taken 2 h after the onset of confinement stress were found to have a significantly elevated plasma bactericidal activity ($P<0.05$) compared with samples from control fish (Fig. 5), with a mean increase in bactericidal activity of approximately 50% across the three vitamin C status groups. However, plasma bactericidal activity was found to be independent of vitamin C status, although there was a tendency for decreased levels of activity in the group fed the low vitamin C diet.

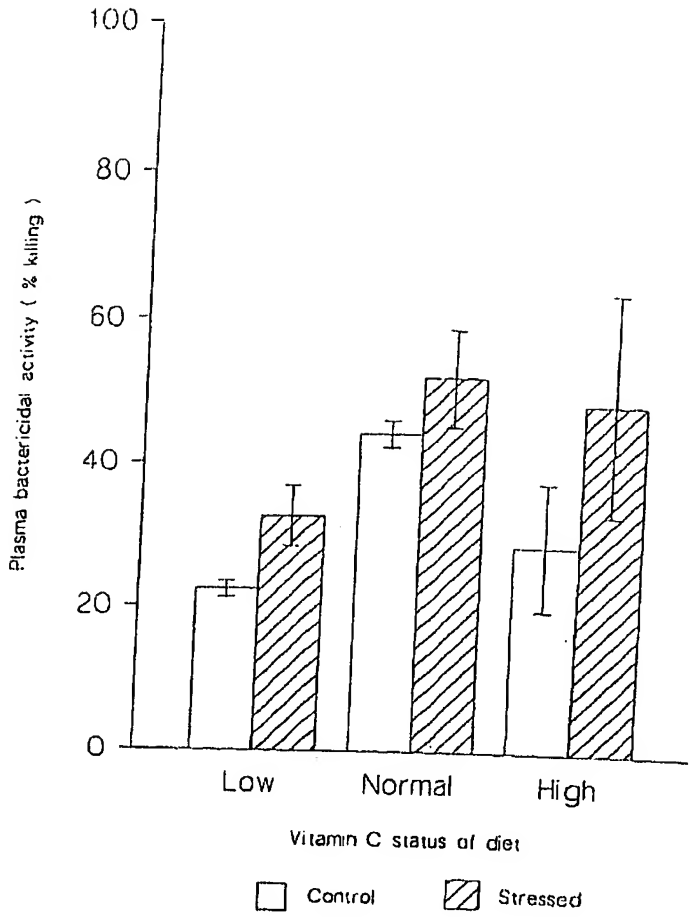

Fig. 5. Bactericidal activity of plasma obtained from controls and 2 h post-stress salmon fed three different levels of vitamin C. Data are expressed as means of 5 fish ± standard error.

TABLE 3

Antibody titres (log_2) from control and stressed (at time of immunisation) salmon fed three different levels of vitamin C measured on days 29, 43 and 64 post-immunisation. Data are means of 5 fish ± standard error

Days post immunisation	Low		Normal		High	
	Control	Stressed	Control	Stressed	Control	Stressed
Day 29	4.4 ± 0.4	5.0 ± 0.3	5.2 ± 0.5	5.8 ± 0.5	5.4 ± 0.2	5.0 ± 0.0
Day 43	9.2 ± 1.1	8.4 ± 0.7	8.6 ± 1.0	6.2 ± 0.4	7.0 ± 1.4	5.0 ± 0.6
Day 64	9.4 ± 1.1	7.0 ± 0.7	7.6 ± 0.4	5.8 ± 0.8	6.6 ± 1.2	5.8 ± 0.7

Specific antibody response

A 2 h confinement stress immediately prior to immunisation with formalin-killed *A. salmonicida* was not reflected in any reduction in serum antibody titres in any of the three vitamin C status groups 29 days post immuni-

sation and there was no evidence of any effect of vitamin C status on antibody titres (Table 3). Forty-three days post immunisation, however, the stressed fish were found to have significantly lower antibody titres ($P<0.01$) compared with the controls, reflecting a mean drop of around 20% in levels of circulating antibody across the three vitamin C status groups. In addition, significantly higher titres were found in the fish fed the low vitamin C diet compared with those maintained on the high vitamin C diet ($P<0.01$). Sixty-four days post immunisation, no significant effects of stress or vitamin C status were apparent in the antibody titres, though the trends were similar to those observed at day 43. Background titres in unimmunised fish from all groups were negligible (<2).

DISCUSSION

Salmon lack the enzyme L-gulonolactone oxidase and therefore have little or no ability to synthesise vitamin C (Dabrowski, 1990). This means that the entire requirement must be provided in the diet. This was apparent in the fish fed the low vitamin C diet where liver and kidney vitamin C concentrations of $<20\,\mu g/g$ were found. Liver levels of $<50\,\mu g/g$ are considered to represent deficiency in Atlantic salmon (Lall et al., 1989), but there was no evidence of scoliosis, lordosis or loss of appetite in the low vitamin C status group in the present experiment. This lack of a clinical effect was also reflected in the growth rate of this group which was comparable to that achieved by the normal and high vitamin C status groups. It is possible that higher specific growth rates may have resulted in more pronounced clinical effects. Previous studies have shown that tissue levels of vitamin C accurately reflect dietary input, with no significant evidence of a saturation plateau emerging up to a maximum ration level of 2.75 g/kg diet (Lall et al., 1989; Hardie et al., 1991). In this investigation, liver and kidney vitamin C concentrations were similar in both the low and normal vitamin C status groups, but liver concentration was significantly lower than that found in the kidney in the group fed the high vitamin C diet. This suggests that the liver may begin to become saturated if vitamin C levels are increased above 2.75 g/kg diet.

The relationship between cortisol and plasma glucose is poorly documented, but available evidence suggests that cortisol may help to maintain high levels of plasma glucose post stress (Leach and Taylor, 1980). If the interrenal level of vitamin C is a limiting factor in the regulation of the biosynthesis of cortisol, one would expect to find a lower level of plasma glucose post stress in the group fed the high vitamin C diet compared with those fed the normal and low vitamin C diets. Evidence that vitamin C is limiting and ameliorates the effects of stress in a dose-dependent manner exists in the poultry literature (Brake et al., 1992). In the present investigation, the magnitude of the hyperglycaemic response was found to be independent of the

vitamin C status of the fish, providing indirect evidence that vitamin C is not involved in retarding the biosynthesis of cortisol. The plasma glucose levels measured in the control and stressed fish in this study are in agreement with those in the literature (Pickering et al., 1982; Möck and Peters, 1990).

The haematological changes resulting from 2 h of confinement stress were found to be minimal, with a slight elevation observed in the number of circulating lymphocytes. A similar elevation in the number of circulating lymphocytes was observed to precede a period of prolonged lymphocytopenia in stressed trout (Pickering et al., 1982).

Respiratory burst activity of phagocytes was found to be reduced by about 40% in the stressed fish, and a reduction has been also reported in stressed bass (Stave and Robertson, 1985), and rainbow trout (Angelidis et al., 1987). This suppression of the respiratory burst was accompanied by a reduction of about 50% in leucocyte bactericidal activity. The production of highly toxic O_2^- radicals during the respiratory burst has been shown to be one of the principal mechanisms by which phagocytes can kill the strain of *A. salmonicida* used in the latter experiment (Sharp and Secombes, 1993).

The mechanism by which cortisol suppresses phagocyte function is not well understood. In mammalian systems, cortisol is known to reduce the membrane permeability of leucocytes and therefore prevent the free exchange of many metabolic precursors including glucose (Munck, 1971) and nucleosides (Makman et al., 1968) as well as exerting deleterious effects on ATP production (Nordeen and Young, 1976) and promoting catabolism of protein (MacDonald and Cidlowski, 1982). It is likely that some of these effects of cortisol occur in fish phagocytic cells, any one of which or a combination thereof could account for a suppression of phagocyte function. In the present study, the vitamin C status of the fish had no effect on the magnitude of phagocyte respiratory burst activity or bactericidal activity, nor was there any evidence of vitamin C-mediated amelioration of the stress-induced suppression of these immune parameters. This latter observation provides further evidence that high levels of vitamin C in the interrenal tissue cannot retard biosynthesis of cortisol in fish.

Leucocyte migration appeared to be independent of vitamin C status in this investigation. This is the first report of the effect of vitamin C on leucocyte migration in fish, and differs from studies involving mammals in which leucocyte migration is significantly compromised in animals fed low levels of vitamin C (Johnston and Huang, 1991). Leucocyte migration was also unaffected by stress, in contrast to a previous study involving plaice injected with cortisol (MacArthur and Fletcher, 1985). The lack of stress-induced suppression of leucocyte migration means that no conclusions may be drawn from these data concerning the role of vitamin C in steroidogenesis, but there is no obvious benefit to be gained from feeding elevated levels of vitamin C in the context of this immune parameter.

Plasma bactericidal activity was found to be enhanced in the stressed fish. This may be attributable to elevated levels of lysozyme which can occur in response to some stressors (Möck and Peters, 1990), since the lysozyme of salmonids has been shown to be bactericidal for *A. salmonicida* (Grinde, 1989). There was no significant effect of vitamin C on plasma bactericidal activity as has also been reported by Lall et al. (1989), although there appeared to be reduced levels of bactericidal activity in the fish fed the low vitamin C diet. Since complement levels have been shown to be reduced in sera from salmon fed a low vitamin C diet in a comparable study (Hardie et al., 1991), this could account for this phenomenon. The enhanced plasma bactericidal activity observed in the stressed fish suggests stress need not always be immunosuppressive, and may explain the increased resistance to *Vibrio anguillarum* observed 24 h post stress in rainbow trout (Maule et al., 1989).

Specific antibody levels 43 days post immunisation were found to be suppressed by stress, as observed in several previous studies (Miller and Tripp, 1982; Maule et al., 1989), although the mechanism by which cortisol suppresses lymphocyte function is not yet fully understood. Mitogen responses and antibody production by lymphocytes from stressed fish are significantly depressed and this loss of responsiveness was due to a direct effect on lymphocytes rather than on macrophages (Ellsaesser and Clem, 1986). Cortisol appears to inhibit antibody production by suppression of the B cell precursor pool. This effect is reversible in the presence of conditioned media, indicating a possible lesion in interleukin production by stressed leucocytes (Kaattari and Tripp, 1987). Significantly elevated levels of antibody 43 days post immunisation in the fish fed the low vitamin C diet compared with those fed high levels differs from studies showing no significant effect (Lall et al., 1989; Hardie et al., 1991), or elevated antibody levels in fish fed high levels of vitamin C (Navarre and Halver, 1989). This may be due to the higher concentration of vitamin C in the high vitamin C diet used here, compared with the other studies mentioned. This study may indicate that long-term maintenance of fish on very high levels of vitamin C may have deleterious effects.

In conclusion, this study has provided more evidence that the kinds of stressor prevalent in the aquaculture industry are indeed immunosuppressive. There was no evidence that vitamin C can ameliorate the down regulation of the immune system that occurs following confinement stress, supporting the theory that vitamin C does not play a fundamental role in regulating the primary stress response in fish (Dabrowska et al., 1991). The lack of any significant increase in immunocompetence in the fish maintained on the high vitamin C diet casts some doubt over the value of feeding elevated levels of this vitamin.

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Short communication

The effect of a short-term mental stressor on neutrophil activation

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Abstract

Twenty-five undergraduates and university staff (15 females, 10 males) volunteered to take part in a study examining the effects of a short-term mental stressor on the activation of neutrophils in peripheral blood, as determined by the oxidative capacity to reduced Nitro-blue Tetrazolium (NBT). Participants were assigned to one of two groups, an experimental group ($n = 17$) and a control group ($n = 8$). Subjects in the experimental group were subjected to a time-constrained mental stressor and finger-stick blood samples were taken on four occasions. Those in the control group did not complete a stressor task and only experienced the four finger-stick blood samples. Heart rate was recorded at 5-min intervals as a general indicator of arousal. Examination of the stained blood samples showed that a short-term stressor resulted in significant increased activation of neutrophils, which returned almost to baseline levels on completion of the experiment. In contrast, the control group's neutrophils showed no significant change in activation throughout. The results support the hypothesis that short-term, acute stressors may activate neutrophils.

Keywords: Stress; Psychoneuroimmunology; Neutrophils; Polymorphonuclear leucocytes; Nitro-blue Tetrazolium (NBT) test

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1. Introduction

Early research into the relationship between stress and illness suggested that potential mediators were the stress hormones, such as cortisol, adrenaline and noradrenaline (Cannon, 1932; Selye, 1956, 1976). Current literature suggests that exposure to hostile conditions or other psychological stressors, initiates the secretion of several hormones, including cortisol, catecholamines, prolactin, oxytocin and renin, as part of the survival mechanism (Van de Kar and Blair, 1999). Increasingly, evidence suggests that in addition to the stress hormones, other factors such as nitric oxide (NO) modulate the immune system (Lopez-Figueroa et al., 1998). Most of the research in this area has focused on components of the specific immune system (i.e. antibody titers), or natural killer cell (NK cell) activity. However, NK cells only represent approximately 1% of the population of leucocytes in blood plasma. Antibodies too are only produced in significant numbers, in response to specific pathogens. In contrast, polymorphonuclear leucocytes (PMN), and in particular neutrophils, represent between 50 and 70% of the total population of leucocytes in blood plasma. By responding rapidly to chemostatic stimuli, neutrophils have the ability to migrate though endothelial cell walls to sites of infection. They are also the most abundant cell population in acute inflammatory responses (Alberts et al., 1994). There have been few reported studies on the potentially damaging effects of stress-induced activation of neutrophils.

PMNs are widely appreciated for their beneficial effects on human health. For example, the potent antimicrobial activity of the neutrophil is now recognised (Alberts et al., 1994). Recently, however, the potentially detrimental effects of neutrophils have been implicated, at least in part, in a number of diseases, that may involve the destruction of tissues. These include adult respiratory distress syndrome, myocardial infarction, rheumatoid arthritis, gout, emphysema, glomerulonephritis, inflammatory bowel disease, asthma, immune vasculitis, neutrophil dermatoses, and thermal injury (Malech and Gallin, 1987).

Several ways exist in which neutrophils might induce tissue injury. The major possibilities include production and release of toxic oxygen metabolites, granular components, or products of arachidonic acid metabolism. Neutrophils may contain as many as 50 proteolytic enzymes, with an overwhelming capacity for destruction (Weiss, 1989). Consequently, when neutrophils are exposed to one or various stimuli, a process occurs which is similar to the reaction following ingestion of bacteria, except that the reaction appears to be directed extracellularly into the surrounding tissues rather than internally into phagocytic vacuoles. Thus an activated PMN leucocyte could damage surrounding tissues.

Many animal models have implicated neutrophils in tissue injury (Schraufstatter et al., 1984; Sinha et al., 1988). Similarly, many animal models have shown a role of oxygen-free radicals in tissue injury. However, whether neutrophil-derived, oxygen-free radicals act directly or whether they act indirectly via proteinases (e.g. by activating proteinases or inactivating antiproteinases) remains unclear. Differences in neutrophil proteinases, as well as the antiproteinases have been noted in different species (Abramson and Wheeler, 1993). Furthermore, caution must be applied in extrapolating animal data to the human situation.

It has been understood for several years that the number and distribution of leucocytes in the blood increases during a physical stressor, such as exercise (Shephard and Shek, 1996). Gleeson and Cave (1992). Gleeson et al. (1993) demonstrated that physical exercise induces leucocytosis from the marginal pools. However, non-physical stressors have now also been shown to influence the number and distribution of leucocytes in the blood. Dhabhar et al. (1996) and Kang et al. (1996, 1997) have found that the mental stress of academic examinations and short-term acute stress is sufficient to induce increases in the number and distribution of leucocytes. These changes were found to be both rapid and reversible. Kang et al. (1996) found that superoxidase production in neutrophils increased in those undertaking examinations.

Activated neutrophils can be identified in blood samples using the Nitro blue Tetrozolium (NBT)

reduction assay (Chanine et al., 1996). NBT is a soluble compound which, in the presence of reducing agents, changes from a yellow to a dark blue formazan pigment. This test measures superoxide anions released during the oxidative burst by neutrophils (Chanine et al., 1996). Recent studies have demonstrated that the NBT reduction assay is a reliable measure of activated neutrophils in whole blood (Delano et al., 1997; Takase et al., 1999). Using this test it is possible to distinguish between activated neutrophils from non-activated neutrophils by observing changes in visual appearance, when viewed under a light microscope. Activated neutrophils are distinguished from their non-activated counterparts by blue–black stained granules in the cytoplasm.

Research has shown that increases in cardiovascular function may occur in response to psychological stressors (Gregg et al., 1999). Consequently, measures of heart rate may be considered an appropriate, albeit somewhat general indicator of a stress response. The procedure in this experiment requires the obtaining of fingerstick blood samples at four times. The very nature of this procedure is potentially stress inducing. The inclusion of a control group, who did not receive the stressor task, but who were required to provide blood samples, allowed for an investigation of this possibility. The stressor task used was the advanced progressive matrices, set II of Ravens (1994). A written visual–spatial task comprising of 36 questions, on which a 15-min time constraint was imposed. A pilot study had shown that participants would be unable to complete the task in the time allocated to them, thus increasing the likelihood of a stress response. The aim of this study was to examine the effect of a short period of stress (15 min) on the activity of neutrophils. It was hypothesised that a short period of acute psychological stress will increase the percentage of activated neutrophils.

2. Method

2.1. Design

A repeated measure design was implemented with two groups; an experimental group and a control group. The independent variable was the extent to which physiological arousal was induced by a time-constrained performance test (the stressor). The dependent variable was the percentage of activated neutrophils. It was predicted that significantly more neutrophils would be activated immediately after administration of the performance test (the stressor) than were activated after relaxation.

2.2. Subjects

The sample comprised of 25 undergraduates and university staff. Ten participants were male, aged 20–62 years (mean = 28.8). Fifteen participants were female, aged 20–54 years (mean = 28.7). Four females and four males were randomly assigned to a control group ($n = 8$). The remaining participants ($n = 17$), 11 females and 6 males were assigned to the experimental group

2.3. Procedure: experimental condition

Upon arrival at the laboratory, participants were briefed regarding the nature of the experiment, i.e. examining the effects of task-specific performance on neutrophils activity, and asked to sign a consent form. A heart rate transceiver (Polar, Heart rate monitor) was attached directly to the chest and heart rate was monitored. Participants were seated, asked to make themselves comfortable, close their eyes and breathe orthonasally. This procedure was carried out for a period of 5 min, in order to minimise possible stress levels experienced prior to, or upon arrival, at the laboratory. At the end of this period baseline heart rate was recorded and the first blood sample taken (see Section 2.6). Participants were then instructed to complete the stressor task (Raven's Advanced Progressive Matrices task; Ravens, 1994). An answer sheet was provided for this purpose. A 15-min time constraint was imposed for completion of this task. During this period, heart rate was recorded at 5-min intervals. Upon completion of the test, heart rate was recorded again, and a second blood sample was taken. Two further blood samples and heart rate

measures were taken at 5- and 10-min intervals post-test. A thorough debriefing was provided prior to participants departing the laboratory.

Microscopic examination of the samples was undertaken (40 × mag.). Counts of the total number of observed neutrophils (never less than 100 cells), on each slide, for each participant, at each time point, were made. The total number activated was also recorded in each of these counts. From this data a mean percentage 'activated' was derived, for each participant, at each measurement epoch.

2.4. Procedure: control condition

Participants in the control condition underwent the same procedure as those in the experimental condition but were not required to complete the stressor task. Instead they were asked to sit quietly for the remaining 25 min of the experiment. Heart rate monitoring and blood sampling was carried out in accordance with the procedure for the experimental group. At the end of the experiment, participants were debriefed and the samples were examined as described above.

2.5. Sample procedure

Finger-stick blood samples were taken on four occasions: initial baseline; immediately upon task-completion; 5-min post-task completion; and 10-min post-task completion. The finger-stick was taken from a cleaned, dry fingertip, using a Softclick and lancet (Boehringer Mannheim, Soft click pro. and lancets). The first drop of blood was wiped away (for 'good practice' to eliminate possibly damaged blood cells caused by the finger-stick). Little or no pressure was applied to the finger to obtain the samples. Samples were collected in a Gilson pipette (20 μl), mixed with 20 μl of Nitro-blue Tetrazolium and smeared gently onto two glass slides

2.6. Blood sample protocol

The staining of the blood samples with reagents was necessary, soon after collection. Finger-stick blood samples (20 μl) were mixed with an equal quantity of Nitro-blue Terazolium (NBT) (Sigma Diagnostics cat. no. 840-10) diluted at 1 mg/ml with phosphate buffered saline 0.9%. These samples were placed on microscope slides, incubated at 37°C for 10 min, and then air-dried (two samples were made for each participant at each time). Once dry the samples were flooded with Accustain Wright stain Modified (0.3% w/v buffered at pH 6.9 in methanol. Batch no. 096 H4372) and rinsed with distilled water.

3. Data analyses

Data are expressed as means ± S.D. Statistical analysis was performed with the statistical package for social sciences (SPSS Inc., Chicago, USA). Data comprised pairs of means from two groups (control and experimental) at set time points (four for activation and six for heart rate). Multivariate one-factor analyses of variance were carried out, looking at contrasts for individual components. In order to test for violations of ANOVA assumptions the data underwent the Box's M test, Levene's test and Bartlett's test of sphericity (Tabachnick and Fidell, 1996). Statistical significance was assumed at $P < 0.05$.

4. Results

Both the activation and the heart rate data were subjected to exploratory data analysis to insure robustness and normality of the data. In both cases the results of Box's M and Levene's test were not significant, Bartlett's test of sphericity was significant ($P > 0.001$). Therefore it was deduced that ANOVA assumptions had not been violated.

4.1. Activation data

The data obtained from the NBT test revealed that the mean percentage of activated neutrophils increased in the experimental group when compared to the control group. No significant differences were found between the sexes or for the age of the participants (data not included). Table

Table 1
The mean percentage of activated neutrophils at the four measuring time points[a]

Measuring time points	After a period of relaxation (baseline)		At the end of the stressor period		5 min after the stressor period		10 min after the stressor period	
	Mean%	S.D.±	Mean%	S.D.±	Mean%	S.D.±	Mean%	S.D.±
Experimental group ($n = 17$)	37.56	4.55	59.99	4.19	46.70	4.57	40.87	3.36
Control group ($n = 8$)	38.35	4.90	37.47	5.54	36.75	5.38	39.80	5.53
Significance	NS		$P < 0.001$		$P < 0.001$		NS	

[a] Comparing the experimental group with the control group and also showing ± standard deviation (S.D.).

1 summarises the comparisons of the means and standard deviations of the two groups. Statistical analysis of the means reveals a significant change in activation and a significant change between the groups during the period of the experiment ($F = 556.74$; d.f. 4,20; $P < 0.001$) ($F = 130$; d.f. 4,20; $P < 0.001$). Analyses of the pairs of means from each testing point shows that there is no significant difference between the groups after relaxation (baseline). However, as predicted, the mean percentage activation had significantly increased by the end of the stressor ($F = 128.12$: d.f. 1,23; $P < 0.001$). There is still a significantly elevated level of activation in the experimental group 5 min after the stressor ($F = 23.09$; d.f. 1,23; $P < 0.001$). Levels of activation have returned to near baseline 10 min after the stressor and are not significant.

4.2. Heart rate data

No significant differences were found between the sexes or for the age of the participants in this experiment. Table 2 compares the mean and standard deviation of the heart-rate data of the two groups. It can be clearly seen that the heart rates of the experimental group increased during the stressor task. Statistical analysis of the data reveals that there was a significant change in heart rate between the groups and during the period of the experiment ($F = 246.81$; d.f. 6,18; $P < 0.001$) ($F = 9.42$; d.f. 6,18; $P < 0.001$). Analyses of the pairs of means at each measuring point reveal where the significant differences lie. There was no significant difference at the first time point (baseline). However, during the stressor task there was a significant increase; at 5 min ($F = 4.43$; d.f. 1,23; $P = 0.05$), at 10 min ($F = 9.05$; d.f. 1,23; $P < 0.05$), and at the end of the stressor (15 min) ($F = 9.05$; d.f. 1,23; $P < 0.05$). Comparison of the pairs of means 5 and 10 min after the stressor is not significant. Therefore, it can be deduced that as predicted the stressor task increased heart rate, which returned to normal a short time after the task.

5. Discussion

The results revealed a marked increase in the activation of neutrophils in response to a short-term acute psychological stressor. In contrast, measures obtained in the control condition showed there was little or no change in neutrophil activation. Consequently, these findings support the hypothesis that a short period of acute psychological stress, as provided by the Raven's test, is sufficient to increase the activation of neutrophils. Also, as expected, an examination of the heart rate data clearly showed an increase in heart rate during the stressor task. Whilst, no significant changes were observed in heart rate amongst subjects in the control condition. The results support the proposition that it

Table 2
Comparing the means and standard deviation of the heart-rate data of the two groups[a]

Measuring time points	After a period of relaxation (baseline)		5 min into the stressor task		10 min into the stressor task		At the end of the stressor task (15 min)		5 min after task (20 min)		10 min after task (25 min)	
	HR	SD	HR	SD	HR	SD	HR	SD	HR	SD	HR	SD
Experimental group ($n = 17$)	79.65	11.74	90.65	11.08	94.00	10.63	93.24	10.81	80.71	9.67	79.41	9.90
Control group ($n = 8$)	84.25	13.09	81.10	9.23	80.80	9.41	80.88	11.04	77.10	8.48	78.00	9.38
Significance	NS		$P < 0.05$		$P < 0.05$		$P < 0.05$		NS		NS	

[a]Showing that the stressor task significantly increased HR in the experimental group, whilst the control group remained constant throughout the experimental period. HR, mean heart rate (b.p.m.); NS, no statistical significance (difference).

was the stressor task not the finger-stick procedure that induced the increase in percentage activation of the neutrophils. There was a high degree of variability between individuals for heart rate measurements, possibly due to the situation. However, without exception all participants in the experimental group recorded an increase in heart rate during the stressor task. In contrast those in the control group remained at or near baseline throughout.

Dhabhar et al. (1996) suggested that specific receptors expressed on the surface of the neutrophil may be responsible for increased cell numbers in peripheral blood. Since neutrophils have a number of receptor types expressed on their surface, in particular beta-adrenergic, these may be responsible for the increased activation. Gregg et al. (1999) proposed that different types of stressors, psychological or physical, induce different physiological responses through adrenergic receptors. The type of stressor used in this investigation is 'beta-adrenergic', and is characterised by the relaxation of smooth muscle, but increasing contraction of cardiac muscle. Thus, promoting an increase in cardiac rate. Passive physical stressors, such as the cold pressor test (immersion of the forearm in cold water) (Gregg et al., 1999), are described as alpha-adrenergic, causing contraction of smooth muscle; a vasoconstriction effect (Gregg et al., 1999). It is reasonable therefore to deduce that beta-adrenergic receptor sites on neutrophils may be involved in their activation. However, further research needs to be undertaken to elucidate these factors. Indeed, many other contributory factors may be involved. Kang et al. (1997) observed fluctuations in the levels of cytokines, interferons and interleukins during stressors. These to may contribute to the mechanism for the stress-activation of neutrophils. Sendo et al. (1997) reported that psychophysical stress modulates the apoptosis of neutrophils. It is suggested that prolonging their active life, facilitates the bactericidal activity of the cell. However, inhibition of neutrophils apoptosis may in turn accelerate tissue toxicity by these cells during inflammation (Sendo et al., 1997). Further research into this area is necessary before it is possible to obtain a thorough understanding of the relationship between neutrophils activation and stress.

Following activation, the neutrophil's membrane-bound nicotine adenine dinucleotide phosphate (NADPH) oxidase system begins to generate large quantities of superoxide anion. Almost simultaneously, neutrophil granules fuse with the external membrane at sites of activation and toxins are released into the extracellular medium. As a result, host-tissues are directly attacked by PMN leucocyte-derived toxins (Weiss, 1989).

In conclusion, there has been a paucity of research into the role of neutrophils in health and disease. Our research is the first reported study on the effect of a short-term psychological stressor on PMN leucocyte activation. We have observed that a short-term stressor lasting only 15 min is sufficient to activate the most abundant leucocyte in the body, the neutrophil. The neutrophil plays a pivotal role in protecting the body against disease. Once activated, the neutrophil would be unable to respond to opportunistic infections, e.g. invading bacteria, thus would render the body more susceptible to disease, as well as possible tissue damage from released enzymes and metabolites.

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